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STEM CELLS FOR CARDIAC REGENERATION

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Stem cells for cardiac regeneration

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The day science begins to study non-physical phenomena, it will make more progress in one decade than in all the previous centuries of its existence.

Nikola Tesla

Mojoj porodici

ABSTRACT

Recent studies have demonstrated a turnover of cardiomyocytes throughout the adult life. Whether this regenerative potential is due to proliferation and differentiation of resident stem cells or to the dedifferentiation of adult cardiomyocytes remains unclear. Generation of new cardiomyocytes is critical for cardiac repair following myocardial injury; however the mechanisms by which injury responses modulate differentiation and proliferation of cardiomyocyte progenitors is not clear.

In Study I, we show that sublethal apoptotic stimuli modulate the differentiation of mouse embryonic stem cells (ESC) into cardiomyocytes through a caspase-dependent mechanism. This indicates a direct link between caspase activation and initiation of the cardiogenic programs. Further unfolding of these mechanisms may have significant implications for the understanding of the dynamics of cardiac regeneration after myocardial injury involving both exogenous and endogenous cell sources.

The intrinsic regenerative capacity of human fetal cardiac mesenchymal stromal cells (MSCs) has not been fully characterized. In Study II our aim was to establish a culture protocol for large-scale expansion of human fetal cardiac MSCs with characteristics of cardiovascular progenitor cells. By culturing the cardiac MSCs on defined laminin (LN)-based substrata in combination with stimulation of the canonical Wnt/β-catenin pathway we could generate multi-potent cells which could differentiate into endothelial, smooth muscle cells, and spontaneously beating cardiomyocytes. The expanded cardiac MSCs stained positive for the progenitor markers: Pdgfr-α, Isl1, and Nkx2.5, and subpopulations also expressed: Tbx18, Kdr, c-Kit, and Ssea-1. Our protocol for large-scale culture of human fetal cardiac MSCs enables future exploration of the regenerative functions of these cells in the context of myocardial injury *in vitro* and *in vivo*.

Subsequently, in Study III we performed bioenergetic and metabolic profiling of human fetal cardiac MSCs as a tool for investigating metabolic changes in embryonic hearts during development. The critical role of metabolism in the active control of cell renewal and lineage specification has recently come into focus, and Wnt has been proposed as one of the signaling pathways which links energy metabolism to cell fate decision. In the MSCs derived from hearts of different gestational age, we observed an increase in oxidative metabolism during first trimester of development. Finally, stimulation of the canonical Wnt/β-catenin signaling pathway in hypoxia appeared to enhance long-term MSC expansion, and resulted in a cell population with higher cardiogenic differentiation potential. Our findings suggest that manipulation of metabolic signals may facilitate endogenous stem cell activation, tissue renewal and induction of cytoprotective properties.

LIST OF PUBLICATIONS

I. Sublethal Caspase Activation Promotes Generation of Cardiomyocytes from Embryonic Stem Cells
Ivana Bulatovic*, Cristian Ibarra*, Cecilia Österholm, Heng Wang, Antonio Beltrán-Rodríguez, Manuel Varas-Godoy, Agneta Månsson-Broberg, Per Uhlén, András Simon, Karl-Henrik Grinnemo
PLoS One. 2015; 10(3):e0120176

II. Wnt/β-Catenin Stimulation and Laminins Support Cardiovascular Cell Progenitor Expansion from Human Fetal Cardiac Mesenchymal Stromal Cells
Agneta Mansson-Broberg, Sergey Rodin, **Ivana Bulatovic**, Cristian Ibarra, Marie Lofling, Rami Genead, Eva Wardell, Ulrika Felldin, Carl Granath, Evren Alici, Katarina Le Blanc, C.I. Edvard Smith, Alena Salasova, Magnus Westgren, Erik Sundstrom, Per Uhlen, Ernest Arenas, Christer Sylven, Karl Tryggvason, Matthias Corbascio, Oscar E. Simonson, Cecilia Osterholm and Karl-Henrik Grinnemo
Stem Cell Reports. 2016; 6(4):607-17

III. Metabolomic Profiling of Human Fetal Cardiac Mesenchymal Stromal Cells
Ivana Bulatovic, Clifford Folmes, Ivan Vuckovic, Song Zhang, Ryounghoon Jeon, Ulrika Felldin, Marie Lofling, Cecilia Osterholm, Andre Terzic, Karl-Henrik Grinnemo
Manuscript

*These authors contributed equally

PUBLICATIONS NOT INCLUDED IN THIS THESIS

Human fetal cardiac progenitors: The role of stem cells and progenitors in the fetal and adult heart.
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Best Pract Res Clin Obstet Gynaecol. 2016; 31:58-68.

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J Cytol Histol 2013; 4. 192

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IgM phosphorylcholine antibodies inhibit cell death and constitute a strong protection marker for atherosclerosis development, particularly in combination with other auto-antibodies against modified LDL.
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Results in Immunology. 2012 8;2:13-8.

CONTENTS

LIST OF ABBREVIATIONS

AMI	Acute myocardial infarction
BM-MSCs	Adult bone marrow mesenchymal stromal cells
BM-MNCs	Adult bone marrow mononuclear cells
c-Kit	Tyrosine kinase receptor,stem cell growth factor receptor
cMSCs	Cardiopoietic mesenchymal stromal cells
EB	Embryoid bodies
ESC	Embryonic stem cells
EMT	Epithelial-to-Mesenchymal Transition
ErbB4	Erb-B2 receptor tyrosine kinase 4
FHF	First heart field
Gata4	GATA binding factor 4
GHMT	Gata4, Hand2, Mef2c, and Tbx5
Hif	Hypoxia-inducing factor
IGF-1	Insulin-like growth factor-1
iPSC	Induced pluripotent stem cell
Isl1	LIM/homeodomain transcription factor Islet-1
MSCs	Mesenchymal stromal cells
Nkx2.5	NK2 transcription factor related, locus 5
Oct	Octamer-binding transcription factor
OxPhos	Oxidative phosphorylation
ROS	Reactive oxygen species
Pdgfrα	Platelet-derived growth factor receptor alpha
SHF	Second heart field
Ssea-1	Stage-specific embryonic antigen-1
Sca-1	Stem cell antigen-1
TnT	Troponin T
Wnt	Wingless-related integration site
α-MHC	Alpha myosin heavy chain
α-SMA	Alpha smooth muscle actin

1 INTRODUCTION

1.1 HEART DEVELOPMENT

The heart is one of the first organs that develop during embryogenesis and the first organ that displays function. During gastrulation, the process in which the three germ layers of an embryo are formed, a subset of epiblast cells moves as a sheet to the primitive streak and undergoes an epithelial-to-mesenchymal transition (EMT) (Gould *et al.* 2013). The heart is derived from the mesodermal germ layer, which is specified into the cardiac mesoderm through the interaction of inductive and inhibitory signals from the adjacent endoderm and ectoderm (Figure 1)(Srivastava 2006). These signals include Wingless-related integration site (Wnt), fibroblast growth factor (Fgf), and transforming growth factor-β (Tgf-β) pathways leading to the activation of early cardiomyocyte transcriptional programs (Marvin *et al.* 2001, Harvey 2002, Buckingham *et al.* 2005, Lopez-Sanchez and Garcia-Martinez 2011, Brade *et al.* 2013, Spater *et al.* 2014). The next step in cardiogenesis is the specification and differentiation of progenitor cells through the development of specific heart fields (Buckingham *et al.* 2005, Kelly *et al.* 2014). The first heart field (FHF) forms the cardiac crescent in the anterior splanchnic mesoderm at approximately week 2 of human gestation (Brade *et al.* 2013, Kelly *et al.* 2014). In the third week of human gestation, the cardiac crescent fuses at the midline, and it forms the linear heart tube, which starts to beat around embryonic day 22 (Brade *et al.* 2013, Kelly *et al.* 2014). Subsequently, during week 4, the linear heart tube undergoes rightward looping (Zaffran *et al.* 2004), and it grows rapidly through cell proliferation and recruitment of subpharyngeal cells to the arterial and venous poles (Tirosh-Finkel *et al.* 2006, van den Berg *et al.* 2009). The cells originating from the pharyngeal mesoderm migrate and enter the FHF-derived heart tube, differentiating into progenitors of the second heart field (SHF) (Figure 1) (Mjaatvedt *et al.* 2001, Waldo *et al.* 2001, Cai *et al.* 2003, Kelly *et al.* 2014). SHF progenitors are defined by their expression of the LIM-homeodomain transcription factor Islet-1 (Isl1), and they contribute to the atria, outflow tract (OFT), and the right ventricle, whereas the left ventricle mainly seems to be derived from the FHF cells, which are marked by their expression of T-box transcription factor 5 (Tbx5), NK2 homeobox 5 (Nkx2.5), and the ion channel

hyperpolarization-activated cyclic nucleotide-gated channel 4 (Hcn4) (Cai *et al.* 2003, Chien *et al.* 2008, Genead *et al.* 2010, Spater *et al.* 2013, Zhuang *et al.* 2013). A recent study explored how different levels of Tbx5a and pituitary homeobox 2 (Pitx2) contribute to the development of the FHF and the SHF of a Zebrafish, where blocking of their respective actions led to heart dysfunction (Mosimann *et al.* 2015). These findings suggest that FHF and SHF progenitors may give rise to cardiomyocytes with distinctive physiological properties (Mosimann *et al.* 2015).

The early embryonic heart tube consists of two cell layers, the endocardium and the myocardium. The third layer, the epicardium, is derived from the proepicardium, which in turn arises from the coelomic mesenchyme of the septum transversum roughly at embryonic day 21 (Manner *et al.* 2001). The embryonic proepicardial progenitor cells differentiate into cardiac fibroblasts, coronary vasculature, and to some extent into cardiomyocytes (Cai *et al.* 2008, Zhou *et al.* 2008).

The fourth progenitor population involved in cardiogenesis consists of the cranial neural crest cells, and it arises from the dorsal neural tube (Keyte and Hutson 2012, Brade *et al.* 2013, Kelly *et al.* 2014). The neural crest cells are crucial for the septation of the OFT, the formation of heart valves, and the full parasympathetic innervation of the heart (Keyte and Hutson 2012, Brade *et al.* 2013, Kelly *et al.* 2014).

Through complex interactions of FHF and SHF progenitors with proepicardial and cranial neural crest cells, the fetal heart is septated into four defined chambers, and it connects to the aorta and the pulmonary trunk approximately during gestational week 7 (Yi *et al.* 2010, Brade *et al.* 2013, Kelly *et al.* 2014).

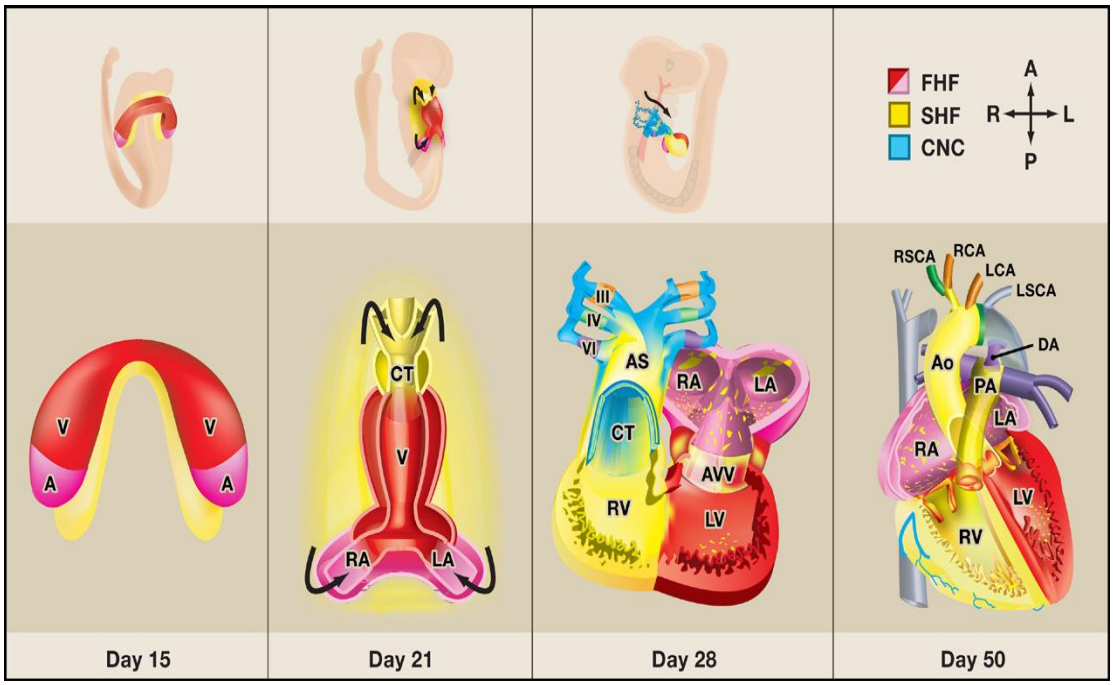

Figure 1. Mammalian Heart Development

FHF cells form a crescent shape in the anterior embryo with SHF cells medial and anterior to the FHF (Second panel). SHF cells lie dorsal to the straight heart tube and begin to migrate (arrows) into the anterior and posterior ends of the tube to form the right ventricle (RV), conotruncus (CT), and part of the atria (A) (Third panel). Cardiac neural crest (CNC) cells also migrate (arrow) into the outflow tract from the neural folds to septate the outflow tract and pattern the aortic arch arteries (III, IV, and VI) (Fourth panel). The next step is septation of the ventricles, atria, and valves (AVV). V, ventricle; LV, left ventricle; LA, left atrium; RA, right atrium; AS, aortic sac; Ao, aorta; PA, pulmonary artery; RSCA, right subclavian artery; LSCA, left subclavian artery; RCA, right carotid artery; LCA, left carotid artery; DA, ductus arteriosus. Reprinted with the permission from the publisher of (Srivastava 2006) http://dx.doi.org/10.1016/j.cell.2006.09.003

1.2 CARDIOVASCULAR PROGENITORS

In order to define cardiovascular progenitors, it is necessary to identify their origin in the embryonic heart and to organize them within the hierarchy of cardiogenesis. Only when we have a reliable map of fetal cardiovascular cell lineages can we look for the presence of endogenous stem cells and progenitors in the adult heart.

A common primordial cardiovascular progenitor that gives rise to progenitors of both the FHF and SHF has been characterized by the expression of transcription factor brachyury (Bry), a member of the T-box family of genes, shown to be critical for mesoderm formation (Showell *et al.* 2004, Kattman *et al.* 2006, Yi *et al.* 2010).

Kattman and colleagues used Bry and kinase insert domain receptor (Kdr), also known as fetal liver kinase 1 (Flk-1), which encodes vascular endothelial growth factor receptor 2, to identify a population of cells with cardiovascular potential (Figure 2) (Kattman *et al.* 2006, Yang *et al.* 2008) during embryonic stem cells (ESCs) differentiation. Individual Bry+Kdr+ cells generated colonies capable of differentiating into cardiomyocytes, endothelial cells, and vascular smooth muscle cells (Kattman *et al.* 2006, Yang *et al.* 2008). Some colonies expressed SHF marker Isl1, whereas others were positive for Tbx5, a marker linked to FHF (Takeuchi *et al.* 2003, Kattman *et al.* 2006, Yang *et al.* 2008). The next marker in the embryonic cardiovascular hierarchy, which may identify an early progenitor population that precedes the separation of the first and second heart lineages, is the transcription factor mesoderm posterior 1 (Mesp1). This factor seems to be important for the activation of the cardiogenic transcription factors: Nkx2.5, Gata-binding protein 4 (Gata4), myocyte enhancer factor 2c (Mef2c), and Isl1 (Saga *et al.* 1999, Bondue *et al.* 2008).

The expression of Nkx2.5 is indispensable for the development of ventricular cardiomyocytes and characterizes FHF- and SHF-derived cells committed to the cardiomyogenic fate (Figure 2) (Stanley *et al.* 2002, Wu *et al.* 2006). Another early marker, which may be used for sorting early cardiac progenitors, is stage-specific embryonic antigen-1 (Ssea-1) (Blin *et al.* 2010). Blin and colleagues have derived these cells from human ESCs and induced pluripotent stem cells (iPSCs), differentiating them into cardiomyocytes, smooth muscle cells, and endothelium (Blin *et al.* 2010). Recently, we were able to sort out a pure population of cells expressing Ssea-1 from human fetal hearts obtained from abortion material (Elsheikh *et al.* 2013). Isolated cells expressed the pluripotent stem cell marker Oct4 as well as the key cardiac transcription factors Nkx2.5, Gata4, Isl1, and Tbx5. Besides expressing the markers of both FHF and SHF, these cells co-expressed the mesenchymal stromal cell markers. Furthermore, the expression of the cardiomyocyte-specific proteins, troponin T (TnT) and actin, was up-regulated following 5-azacytidine treatment (Elsheikh *et al.* 2013).

Hcn4, voltage-gated ion channel, which is a hallmark of the conduction system and appears to specify the cells that predominantly differentiate into atrial and ventricular cardiomyocytes, was recently identified by Spater and colleagues as a specific marker of the FHF (Figure 2) (Spater *et al.* 2013).

The SHF develops from multi-potent cardiovascular progenitors characterized by the expression of the transcription factor Isl1 (Figure 2) (Cai *et al.* 2003, Laugwitz *et al.* 2005, Moretti *et al.* 2006, Bu *et al.* 2009, Genead *et al.* 2010). SHF progenitors migrate from the pharyngeal mesoderm into the heart tube as it grows and undergoes looping (Cai *et al.* 2003, Genead *et al.* 2010). However, Isl1 protein has been detected already at the cardiac crescent stage, suggesting the possibility that Isl1 is expressed in FHF at a very early stage (Prall *et al.* 2007). In the OFT of the human fetal hearts at gestation week 9, Lui and colleagues identified an Isl1+ population of endothelial progenitors expressing VEGF-A receptors (Lui *et al.* 2013). Additionally, Vedantham et al. have recently demonstrated that Isl1 is a regulator of sino-atrial node development (Vedantham *et al.* 2015). Thus, in contrast to Hcn4, Isl1 expression may identify a transient progenitor state important for heart lineage diversification that rapidly converges into the vascular endothelial cell, myocardial and conduction system cell lineages, followed by down-regulation of Isl1 upon cardiomyocyte differentiation (Moretti *et al.* 2006, Genead *et al.* 2010). Recently, Jain et al. identified the earliest committed cardiomyocyte progenitor, cardiomyoblast, defined by the Hopx (homeobox gene) expression in cardiac progenitors from the SHF (Wnt-activated Isl+ progenitors) that are localized to the OFT and start expressing Hopx after down-regulating Wnt (Jain *et al.* 2015). In the work by Genead et al, we characterized the distribution and electrophysiological properties of Isl1-positive progenitor cells in the first-trimester human fetal heart (Genead *et al.* 2010). At gestational weeks 5 to 10, Isl1+ cells were mainly detected in the OFT, but also in the atria and the right ventricle, with certain clusters co-expressing the cardiomyocyte marker TnT. Interestingly, most of the proliferating cells were Isl1-negative but TnT-positive, showing robust proliferative potential of fetal ventricular cardiomyocytes. When the cells were cultured as spontaneously beating cardiospheres, they expressed Nkx2.5 and TnT, whereas Isl1 was detected only in a minority of cells (Genead *et al.* 2010).

The embryonic proepicardial progenitor cells originating from the mesenchyme, express Tbx18 and Wilms tumor 1 (Wt1) (Figure 2), or semaphorin 3D (Sema3d) and scleraxis (Scx) (Cai *et al.* 2008, Zhou *et al.* 2008, Katz *et al.* 2012). These cells primarily differentiate into coronary smooth muscle and endothelial cells, but early in development, a subset of proepicardial progenitors express Isl1 and Nkx2.5 and may also differentiate into cardiomyocytes (Cai *et al.* 2008, Zhou *et al.* 2008).

Finally, the cranial neural crest cells (CNCs), which are involved in the development of heart valves and the parasympathetic innervation of the heart, are defined by the expression of the Pax3 marker (Figure 2) (Jiang *et al.* 2000).
Even in the adult heart, a few resident Isl1+ cells can be found in the OFT, expressing TnT as a sign of cardiac commitment (Genead *et al.* 2012). Other proposed cardiac progenitors in the adult heart are c-Kit+, Sca-1+, platelet-derived growth factor receptor (Pdgfr)-α+, Ssea-1+, and side population cells respectively (Goodell *et al.* 1997, Hierlihy *et al.* 2002, Beltrami *et al.* 2003, Oh *et al.* 2003, Bearzi *et al.* 2007, Ott *et al.* 2007, Chong *et al.* 2011, van Berlo *et al.* 2014, Santini *et al.* 2016). Characterization of these cells is based upon their surface markers and functional properties.

The definitive proof of the existence of endogenous stem and progenitor cells in the adult heart would be of great value in the cardiac regenerative field. Nevertheless, establishing the relationship of these cells to the embryonic progenitors and arranging them within the heart development hierarchy is challenging, and it requires rigorous lineage-tracing strategies.

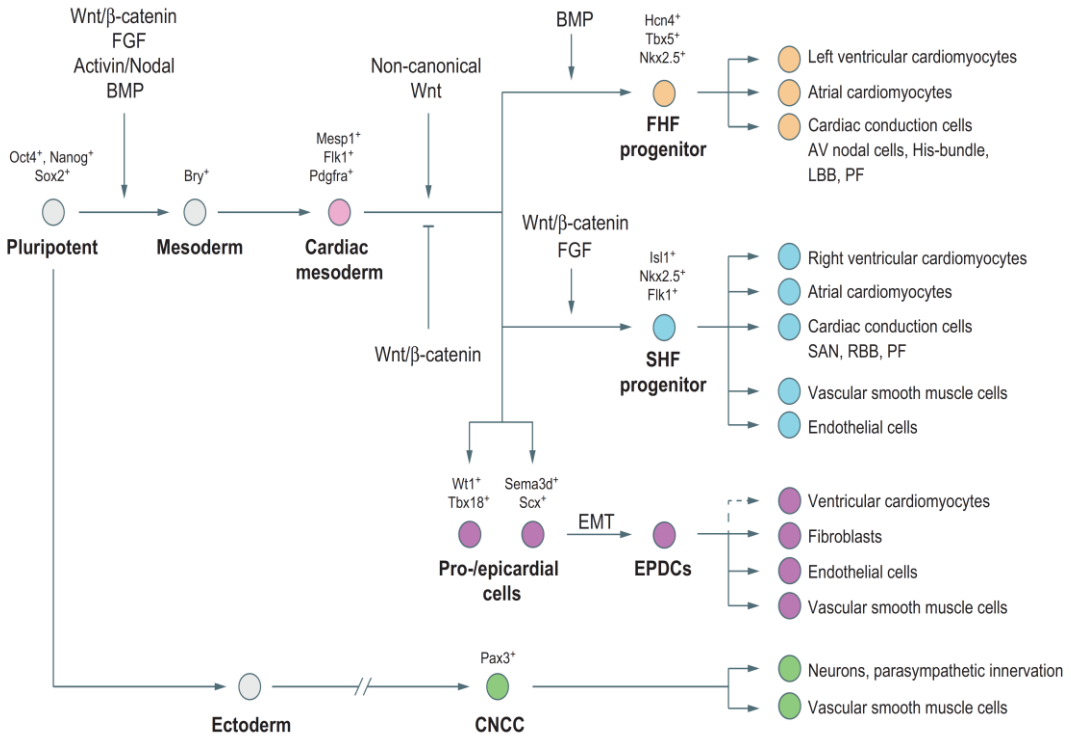

Figure 2. Specification of the cardiac cell lineage.

The stepwise commitment of pluripotent cells via various intermediate stages towards mature cardiac cell types within the heart during development. EPDCs, epicardium-derived cells; EMT, endothelial-to-mesenchymal transition; SAN, sino-atrial node; RBB, right bundle branch; LBB, left bundle branch; PF, Purkinje fibers; AV, atrioventricular. Reprinted with the permission from the publisher of (Spater *et al.* 2014) doi: 10.1242/dev.091538

1.3 ENDOGENOUS CARDIAC STEM CELLS

The cells with the properties of stem and progenitor cells detected in the adult heart are rare, comprising just 0.005-2% of all cardiac cells (Chong *et al.* 2014). These heterogeneous cell populations reside around vessels and among cardiac muscle fibers. Even if some studies suggest certain levels of plasticity of these residing stem cells, their *in vivo* function is less clear and their beneficial effects observed upon transplantation are most probably attributed to paracrine stimulation of angiogenesis and inhibition of apoptosis (Liang *et al.* 2014). Identifying the origin of the adult cardiac stem cells is critical in order to understand how cells with similar properties can be generated from other adult cell sources, which can be used for cardiac regeneration.

1.3.1 c-Kit+ cardiac stem cells

In 2003, a study by Beltrami et al indicated that c-Kit marks rare heterogeneous cardiac-resident adult stem cells, positive for Nkx2.5, Gata4, and Mef2c and negative for hematopoietic lineage markers (Lin^-), and able to self-renew and differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells both *in vitro* and after transplantation (Beltrami *et al.* 2003). Furthermore, c-Kit expression has been reported in cells of bone marrow origin, which enter the heart after injury (Fazel *et al.* 2006, Cimini *et al.* 2007), in postnatal cardiomyocytes (Li *et al.* 2008, Tallini *et al.* 2009), coronary endothelial cells and epicardial cells (Limana *et al.* 2007, Castaldo *et al.* 2008) as well as in dedifferentiated adult cardiomyocytes (Zhang *et al.* 2010). However, other groups have not been able to reproduce the multi-potent capacity of the c-Kit+ cells, but instead suggested a possible transient paracrine effect of these cells after transplantation (Keith and Bolli 2015). The initial positive results reported from the phase I: Stem Cell Infusion in Patients with Ischemic Cardiomyopathy (SCIPIO) trial (Bolli *et al.* 2011), where autologous culture-expanded c-Kit+ cells were delivered intracoronary, are now highly questioned (major concerns over trial's experimental validity). A Phase II study investigating the Combination of Mesenchymal and c-Kit+ Cardiac Stem Cells as Regenerative Therapy for Heart Failure (CONCERT-HF) is presently underway (NCT02501811). Recently, a vigorous debate was initiated by the contrasting findings by Ellison et al (2013) and van Berlo et al (2014) (Ellison *et al.* 2013, van Berlo *et al.* 2014). This led Liu and colleagues to generate *Kit-CreER* mouse line for inducible lineage tracing (Liu *et al.* 2016). The authors were able to follow Kit+ cells from the fetal up to adult heart and concluded that these cells are located within the endocardium and coronary endothelium, maintaining endothelial identity even after myocardial infarction. Also, only very rare cells were found to co-express Kit and cardiomyocyte markers Nkx2.5 or TnT in both healthy and injured hearts (Sultana *et al.* 2015, Liu *et al.* 2016).

1.3.2 Isl1+ cardiac stem cells

The LIM-homeodomain transcription factor Isl1, binds and controls cis-regulatory elements of the insulin gene (Karlsson *et al.* 1990). Isl1 is also expressed in cardiac mesodermal progenitors of the FHF and SHF, with the expression down regulated as these progenitors enter the heart and differentiate into cardiac lineages (Cai *et al.*

2003, Prall *et al.* 2007, Peng *et al.* 2013). A modest expression has also been detected in cardiac neural crest cells by lineage tracing (Engleka *et al.* 2012). Isl1 deletion affects the survival and proliferation of heart progenitors as well as their deployment to the forming heart tube (Cai *et al.* 2003, Laugwitz *et al.* 2005). It has been suggested that Isl1+ cells persist in rodents beyond early heart development into neonatal and adult life, distributed in a pattern that is consistent with a SHF origin (Bu *et al.* 2009, Genead *et al.* 2010). At fetal stages, the majority of the Isl1+ cells co-expressed the cardiomyocyte marker TnT, suggesting a cardioblast identity, and only few of these cells were proliferating (Genead *et al.* 2010). Human fetal Isl1+ cardiovascular progenitors were shown to give rise to the cardiomyocyte, smooth muscle and endothelial cell lineages (Bu *et al.* 2009). The Isl1+ cells are rare in the adult heart, and in mice they are largely confined to the sino-atrial node (Weinberger *et al.* 2012). It is still unclear if Isl1+ cells represent a compelling stem cell population in the adult heart that can be activated for cardiac repair. However, human and mouse ESCs bearing Isl1 reporters, expressing both Isl1 and Nkx2.5, showed trilineage differentiation potential that could be maintained on feeder layers expressing Wnt3a (Qyang *et al.* 2007). These findings suggest that cardiac progenitors marked by Isl1 might be suitable for cell therapy after cardiac injury.
Furthermore, injection of naked DNA encoding Isl1 into the peri-infarct region in mice subjected to myocardial infarction increased left ventricular function and tissue revascularization and reduced fibrosis (Barzelay *et al.* 2012). Isl1 has also been identified as a regulator of EMT in epicardial cells (Bronnum *et al.* 2013), suggesting a promotion of stem cell characteristics in epicardium as another possible regenerative target.

1.3.3 Pdgfrα+ cardiac mesenchymal progenitor cells

A mesenchymal stromal cell-like population has recently been identified in the adult heart expressing both Pdgfrα and Sca1. Based on the ability of these cells to form clonal colonies, this highly heterogeneous population was named cardiac colony-forming units fibroblast (cCFU-F) (Chong *et al.* 2011, Noseda *et al.* 2015). In the embryo, Pdgfrα is expressed in the early cardiac mesoderm (Chong *et al.* 2011, Kattman *et al.* 2011) and in the cardiac neural crest (Hoch and Soriano 2003). In Pdgfrα knockout mice, defects affecting the OFT, the septum, the chambers and the coronary vessels were observed (Soriano 1997). Epicardial specific Pdgfrα knockout

mice show defective EMT and death of cardiac stromal fibroblasts (Smith *et al.* 2011). In the fetal heart, Pdgfrα marks the proepicardial and epicardial-derived cardiac stromal cells as they undergo EMT to enter the interstitial spaces of the chambers as well as the valves (Chong *et al.* 2011). In the adult heart, single-cell expression profiling confirmed that the $Sca1^{+}/Pdgfr\alpha^{+}$ fraction of cardiac interstitial cells probably derives from the epicardium and can be detected in the interstitium and the perivascular niche (Chong *et al.* 2011, Chong *et al.* 2013). Certain colonies were positive for Gata4, Tbx5, and Mef2c, suggesting a cardiac lineage commitment (Furtado *et al.* 2014, Noseda *et al.* 2015). Cultured cCFU-Fs show clonogenicity, long-term self-renewal, and multi-lineage differentiation *in vitro* and *in vivo* (Chong *et al.* 2011, Noseda *et al.* 2015). cCFU-F in both healthy and infarcted hearts were shown to have their lineage origins in the proepicardium and epicardium (Zhou *et al.* 2008, Chong *et al.* 2011, Noseda *et al.* 2015). As such, the cardiac Pdgfrα+ stromal cell fraction may share properties with the epicardium and other mesothelial cells that give rise to stromal and smooth muscle lineages during development (Rinkevich *et al.* 2012). Uchida and colleagues used CRE lineage tracing on a heterogeneous adult cardiac interstitial population expressing Sca1 (Uchida *et al.* 2013), and demonstrated that these cells are unipotent progenitors that differentiate mostly into smooth muscle cells, but also coronary endothelial cells and to some extent cardiomyocytes. However, recently, Sommariva and colleagues reported that cardiac MSCs might also be a source of adipocytes and contribute to fibrosis in arrhythmogenic cardiomyopathy (Sommariva *et al.* 2016). Furthermore, Gli1, the effector of hedgehog signaling, is thought to mark a population of $Pdgfr\alpha^{+}$ perivascular MSCs, which has been related to fibrosis (Kramann *et al.* 2015). The lineage tracing of these cells in hypertensive and pressure overload models of cardiac fibrosis showed that they differentiate into myofibroblasts expressing smooth muscle α-actin (SMA), where Gli1+/SMA+ cells were abundant in the scared area after myocardial infarction (Kramann *et al.* 2015). Cardiac stromal cells might also act as sentinels of cardiac stress and mediate cellular communication between cardiomyocytes, immune cells and other endogenous progenitors through paracrine mechanisms (Ieda *et al.* 2009, Amoah *et al.* 2015). However, additional studies are necessary in order to get better insight into the cardiac interstitial lineage hierarchy and cellular functions in health and disease (Furtado *et al.* 2016).

1.4 PATHWAYS CONTROLLING SPECIFICATION AND DIFFERENTIATION OF EMBRYONIC CARDIAC STEM CELLS

The process of cardiac lineage specification and differentiation is primarily controlled by activating or inhibiting the Activin A, bone morphogenetic protein (BMP), Wnt, Notch, and FGF signaling networks, as well as regulating key cardiac transcriptional factors such as Nkx2.5, Gata4, Tbx5, and the chromatin remodeling protein SMARCD3 (Marvin *et al.* 2001, Harvey 2002, Srivastava 2006, Lopez-Sanchez and Garcia-Martinez 2011, Noseda *et al.* 2011, Kelly *et al.* 2014, Spater *et al.* 2014). The commitment of the Bry+ mesodermal progenitors toward a cardiogenic fate requires the inhibition of canonical and activation of non-canonical Wnt signaling (Gessert and Kuhl 2010). The expansion and differentiation of Nkx2.5 and Isl1+ cardiac progenitors is also dependent on canonical Wnt signaling, and a sequential induction and inhibition of this pathway has led to the development of a robust cardiomyocyte differentiation protocol from embryonic and induced pluripotent stem cells (Lian *et al.* 2012). The Notch, IGF, *Sonic Hedgehog, and* Hippo signaling pathways have also emerged as important regulators in cardiovasculogenesis, and disturbances in these pathways cause a broad spectrum of cardiac anomalies (Xie *et al.* 2012).

Besides the transcriptional signals, which are critical for the activation of cardiac genes, a number of recent reports highlight posttranscriptional regulation by small, noncoding micro-RNAs (miRNAs) as the emerging novel regulator of cardiogenesis and a tool in enhancing the endogenous regenerative potential of the heart (Cordes and Srivastava 2009, Aguirre *et al.* 2014). miR-1 and -499 are shown to be promising candidates in the modulation of cell specification towards myogenic differentiation (Sluijter *et al.* 2010).

Another signaling system involved in cardiogenesis and final development toward a functioning organ is the ephrin/Eph family of tyrosine kinase receptors and ligands, responding to mesodermal induction signals (Wang *et al.* 2004), influencing cell migration, neural cell migration, and cell-to-cell interaction during embryogenesis (Brantley-Sieders and Chen 2004). Furthermore, EphB2 and B4 are distinguished features of arterial and venous endothelial cells (Wang *et al.* 1998), and the lack of or defect expression of these receptors cause severe cardiovascular malformations and defect trabeculation of the ventricles due to poor cell-to-cell interaction between the endothelium and the adjacent tissue during embryogenesis. In

addition, the ErbB–neuregulin signaling system plays an essential role in cardiogenesis. Defect expression of the ErbB family of tyrosine kinase receptors (ErbB2/B4) during embryogenesis causes cardiac malformations and defects of the left ventricular trabeculation (Ozcelik *et al.* 2002). Neuregulin-1 activation of the ErbB4 receptor induces a cascade of intracellular transduction upon stimulation with the ErbB4 ligand neuregulin-1β. Furthermore, neuregulin-1β activation of the ErbB4 receptor is shown to enhance cardiomyocyte differentiation in ESCs (Wang *et al.* 2009, Hao *et al.* 2014, Iglesias-Garcia *et al.* 2015).

The first efforts to differentiate pluripotent stem cells (PSCs) into cardiomyocytes used a strategy of embryoid body (EB) formation, in which the cells differentiate spontaneously into different cellular lineages, yielding a low percentage of cardiomyocytes. Further differentiation protocols were developed, based on increasing insight in the dynamics of signaling pathways that control cardiac development *in vivo*, and succeeded to efficiently generate more than 50% pure populations of cardiomyocyte from PSCs (Laflamme *et al.* 2007, Yang *et al.* 2008, Kattman *et al.* 2011, Lian *et al.* 2012).

Finally, purification methods have been developed based on separation of cells expressing cardiomyocyte specific traits, which yield up to 98% pure populations of cardiomyocytes. These include the expression of the signal regulatory protein alpha (SIRPA) (Dubois *et al.* 2011) and differences in cardiomyocyte glucose metabolism versus lactate metabolism in non-cardiomyocytes (Tohyama *et al.* 2013). A recent study has demonstrated the feasibility to scale up production of human ESC-derived cardiomyocytes, which could integrate after transplantation into non-human primate hearts (Chong *et al.* 2014).

1.5 POSTNATAL CARDIOMYOGENESIS IN MAMMALIAN AND NON-MAMMALIAN VERTEBRATE SPECIES

Our present understanding of cardiac regeneration has been mainly based on studies in the newt and the zebrafish, which are able to fully regenerate hearts after amputation of up to 20% of the ventricle (Laube *et al.* 2006, Jopling *et al.* 2010). Upon injury, cardiomyocytes of the newt and the zebrafish dedifferentiate into progenitor-like cells, characterized by the disassembly of their sarcomeric structure

that reenter the cell cycle and subsequently redifferentiate into mature cardiomyocytes (Laube *et al.* 2006, Jopling *et al.* 2010). Additionally, Lepilina and colleagues suggested that a pool of undifferentiated progenitors is the basis of zebrafish cardiac regeneration (Lepilina *et al.* 2006). Using a genetic fate-mapping strategy, Zhang and coworkers could demonstrate that atrial cardiomyocytes contribute to ventricular regeneration through a step of dedifferentiation (Zhang *et al.* 2013). Following targeted destruction of the ventricle of the zebrafish, atrial cardiomyocytes adjacent to the atrioventricular canal underwent a dedifferentiation process and started to reexpress the cardiac progenitor markers Gata4, Hand2, Nkx2-5, Tbx5, and Tbx20, while proliferating and migrating into the injured ventricle. Moreover, SHF progenitors, resident near the OFT, also seemed to contribute to the ventricular cardiomyocytes. These findings indicate that non-mammalian vertebrate species regenerate ventricular cardiomyocytes through both dedifferentiation and proliferation of cardiomyocytes and, most probably, also by the activation of resident cardiac progenitors.

The regenerative capacity of the mammalian heart is very limited, as cardiomyocytes withdraw from the cell cycle, and become binucleated early on in the neonatal period (Li *et al.* 1996). In mice, especially in the neonatal period, a certain turnover of cardiomyocytes has been shown through the proliferation of already-existing cardiomyocytes (Porrello *et al.* 2011, Senyo *et al.* 2013). Porrello and colleagues have demonstrated that the mouse heart can fully regenerate if amputation of the ventricular apex occurs on the first neonatal day. This remarkable repair seems to be achieved through a mechanism comparable to that of the zebrafish, namely through dedifferentiation and proliferation of existing cardiomyocytes. However, in contrast to the newt and the zebrafish that retain regenerative capacity throughout life, in mice this initial robust regenerative potential is rapidly lost already within the first postnatal week (Porrello *et al.* 2011). Intriguingly, one common trait of the zebrafish and the mammalian fetal heart is that they both reside in a hypoxic environment.

The neonatal mammalian heart is an excellent model to study important factors involved in mammalian regeneration. Meis1, for example, was shown to be a critical regulator of the cardiomyocyte cell cycle in neonatal mice, where Meis1 deletion increased the postnatal proliferative window of cardiomyocytes and reactivated their entry into mitosis (Mahmoud *et al.* 2013). Others used the regenerating neonatal mouse heart to identify a regulator of cell cycle entry, interleukin 13 (O'Meara *et al.* 2015). Recently, Mahmoud et al also demonstrated that pharmacological cholinergic

nerve inhibition impairs neonatal mice heart regeneration, which could be rescued by administration of neuregulin-1 and nerve growth factor (Mahmoud *et al.* 2015). Using a genetic approach to label neural crest lineages, White et al similarly demonstrated that sympathetic denervation with 6-OHDA inhibited neonatal heart regeneration (White *et al.* 2015).

However, the primary post-natal event that results in cardiomyocyte cell-cycle arrest still needs to be identified. One very interesting hypothesis is that transition to the oxygen rich postnatal and the subsequent increase in oxidative metabolism is the upstream signal that triggers cell cycle exit of cardiomyocytes shortly after birth (Puente *et al.* 2014). Indeed, recently, Puente et al. demonstrated that postnatal hypoxemia, ROS scavenging, or inhibition of DNA damage response (DDR) could all prolong the postnatal proliferative window of cardiomyocytes, while hyperoxemia and ROS generators shorten it (Puente *et al.* 2014).

During adolescence, the proliferation of cardiomyocytes is reduced, except for the thyroid hormone surge at postnatal day 15, which activates the IGF-1/IGF1-R/AKT pathway initiating a proliferative burst with a concomitant increase in the number of cardiomyocytes with about 40% (Naqvi *et al.* 2014). However, Alkass et al, using design-based stereology, were not able to confirm these findings and concluded instead that the number of cardiomyocytes is set within the first postnatal week, followed by two waves of non-replicative DNA synthesis (Alkass *et al.* 2015).

In humans, Bergmann and colleagues performed carbon-14 birth-dating studies demonstrating that fewer than 50% of cardiomyocytes are replaced over an entire life span and that the rate of replacement declines with age (Figure 3) (Bergmann *et al.* 2009, Bergmann *et al.* 2015). Several recent reports confirm this finding showing a low (<1% per year) turnover rate in the adult mammalian heart (Mollova *et al.* 2013, Senyo *et al.* 2013, Naqvi *et al.* 2014, Bergmann *et al.* 2015).

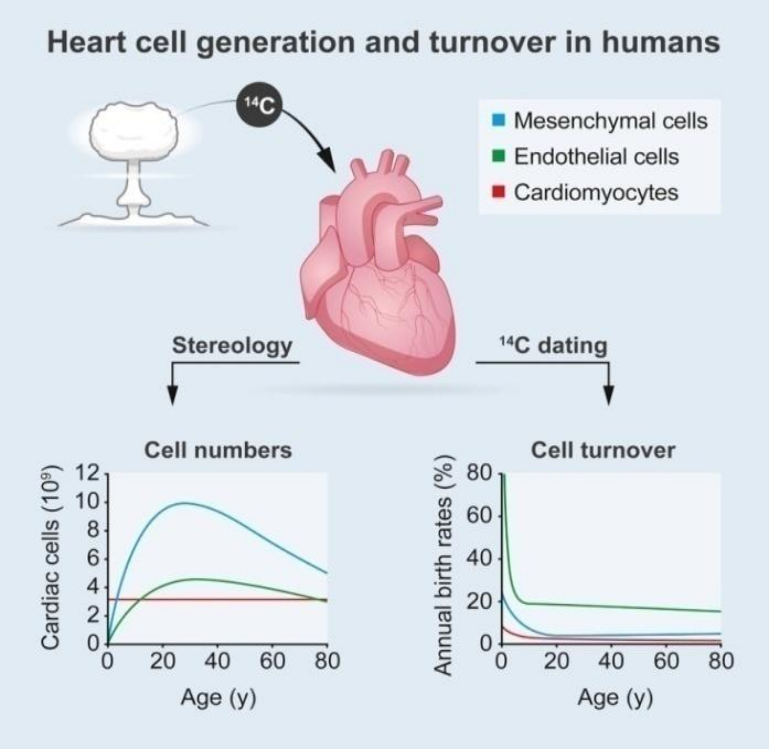

Figure 3. The turnover of cardiomyocytes in humans

A comprehensive analysis of the cell generation and turnover rates shows that cardiomyocytes have a very low turnover rate throughout the human lifespan, with a low turnover rate. Endothelial and mesenchymal cells are exchanged at a high rate. Reprinted with the permission from the publisher of (Bergmann *et al.* 2015) http://dx.doi.org/10.1016/j.cell.2015.05.026

Even if there is no evidence for thyroid hormone-induced proliferation of cardiomyocytes in early adolescence of humans, karyokinesis, division of the nucleus, was observed throughout life, whereas division of the cell cytoplasm (cytokinesis) could not be detected in human hearts after 20 years of life (Mollova *et al.* 2013). From these human studies, we can draw the conclusion that new cardiomyocytes are generated in the postnatal heart but at a slow rate, and that the cellular origin of these cells is still unknown. Importantly, as both Bergmann and Mollova performed studies on presumably normal hearts from individuals without a known history of cardiac disease, the possible increase in the cardiomyocyte turnover rate upon injury is not excluded, as shown in mice subjected to myocardial damage (Senyo *et al.* 2013).

As mentioned previously, the generation of morphologically and functionally different cardiac cells during embryogenesis is achieved by EMT (Kovacic *et al.* 2012). EMT is not only associated with wound healing and organ repair, but also with fibrosis (Kalluri 2009, Kovacic *et al.* 2012). In this regard, a significant difference

between the embryonic and the adult environment is the role of the immune system. Embryos develop in an immune-permissive environment, in contrast with adult tissues in which a robust inflammatory reaction could preclude stem cell differentiation leading to scar formation instead of tissue repair (Santini *et al.* 2016).

1.6 POTENTIAL FOR ENDOGENOUS REGENERATION IN THE ADULT HEART

1.6.1 Contribution of progenitor cells to heart regeneration

As described in the previous section, during aging, the renewal of cardiomyocytes in the mammalian heart is mainly due to the proliferation of existing cardiomyocytes (Senyo *et al.* 2013, Naqvi *et al.* 2014), with some contribution of cardiac progenitors. But what happens after myocardial injury? Hsieh and colleagues were the first to demonstrate the role of cardiac progenitors for myocardial regeneration after myocardial injury (Hsieh *et al.* 2007). They generated a double-transgenic MerCreMer-ZEG mouse model in order to track the fate of cardiomyocytes in a "pulse-chase" fashion. In this transgenic mouse model, the administration of 4-OH-tamoxifen (pulse) induced β-galactosidase (β-gal) to be replaced by green fluorescent protein (GFP) exclusively in cardiomyocytes expressing α-myosin heavy chain (Myh6). Following myocardial infarction or after pressure overload, the ratio between GFP+ and β-gal+ cardiomyocytes was significantly reduced in the infarction border zone and in the pressure-overloaded myocardium, indicating a contribution of cardiac progenitor cells (GFP^- cells) to the cardiomyocyte pool. By contrast, if cardiomyocyte replenishment was done through the proliferation of adult cardiomyocytes, the percentage of GFP+ cells would have been relatively unchanged. In an attempt to further confirm that cardiac progenitors contributed to cardiomyocyte renewal after myocardial injury, c-Kit mRNA was quantitatively measured in the infarcted area up to 7 days post injury, which demonstrated a significant increase of c-Kit mRNA only in the infarcted areas (Hsieh *et al.* 2007).

These findings are supported by a report by Genead et al. (Genead *et al.* 2012), where myocardial infarction but especially ischemia–reperfusion injury but also induced a generalized up-regulation of c-Kit, which also correlated with the concomitant up-regulation of the early cardiomyocyte marker Nkx2.5 (Genead *et al.* 2012). Ischemia–

reperfusion injury also caused a focal up-regulation of Isl1 RNA in the OFT and peri-ischemic regions. Similarly, Smart and colleagues have been able to demonstrate that in adult mice hearts, the expression of the epicardial progenitor markers Wt1 and Tbx18 was significantly increased 7 days after myocardial infarction (Smart *et al.* 2011).

In another study by Ellison and colleagues (Ellison *et al.* 2013), c-Kit+ cardiac stem cells were reported as the primary source for regeneration of cardiomyocytes after myocardial ischemia. Using a diffuse myocardial infarction model in rats, the authors demonstrated that myocardial ischemia induced the proliferation of small mononucleated cardiomyocytes starting at day 3, and during the following 25 days there was a significant increase in the number and size of cardiomyocytes (Ellison *et al.* 2013). *In vivo* genetic cell-fate-mapping studies, tagging the c-Kit+ cardiac stem cells, demonstrated that the majority of the newly formed cardiomyocytes after myocardial ischemia originated from these progenitors. This study, however, has been highly questioned after the contrasting report by van Berlo et al. (van Berlo *et al.* 2014), who generated transgenic mice in which the c-Kit locus was used for lineage tracing. According to this study, the contribution of c-Kit+ cells to cardiomyocyte renewal in aging and injured myocardium was very low, and it should have no functional implication. Similarly, with five independently derived lineage tracing mouse models, Sultana et al found that c-Kit+ cells generate less than 0.05% cardiomyocytes during development, aging, or after a myocardial infarction and ,instead, represent a subpopulation of endothelial cells (Sultana *et al.* 2015).

There also seem to be an age-related heterogeneity among c-Kit+ cells. After myocardial infarction induction in neonate mice hearts, the proliferating c-Kit+ cells adopted both a myogenic and vascular phenotype, whereas infarction of the adult heart resulted in a modest c-Kit+ cell proliferation, and a vascular fate decision (Jesty *et al.* 2012). This may indicate that c-Kit+ cells represent different subpopulations where the real multi-potent cardiovascular progenitor cells disappear with age.

1.6.2 Direct Lineage Conversion

In 1987, a seminal discovery was made showing that ectopic expression of a single skeletal muscle gene, *Myod1*, could convert fibroblasts into skeletal muscle cells (Davis *et al.* 1987). Recently, local delivery of the three transcription factors (Gata4, Mef2c, and Tbx5; together known as GMT) into the infarcted area of mice hearts

induced reprogramming of the cardiac fibroblasts into functional cardiomyocytes (Qian *et al.* 2012).

Addition of *Hand2* to these three factors (GHMT) increased the direct reprogramming efficiency from approximately 7% to 20% (Song *et al.* 2012). To translate this direct reprogramming system from mouse to human, it was discovered that *Myocd* was additionally required (Nam *et al.* 2013). Polycistronic vectors were used to express different ratios of the GMT factors and found that, if *Mef2c* expression is increased compared with *Gata4* and *Tbx5*, then reprogramming efficiency of fibroblasts into cardiomyocytes could be increased five-fold (Wang *et al.* 2015).

1.7 THE ROLE OF CASPASES IN CARDIAC DIFFERENTIATION

The mechanism by which heart injury modulates the activation of cardiac stem cells is still poorly understood, where several studies suggest a role of released paracrine factors from cardiomyocytes, changes in the metabolic environment, exosome signaling, pro-inflammatory cytokines as well as recruitment of bone marrow-derived MSCs (BM-MSCs) (Steinhauser and Lee 2011). Still, an unresolved question is whether different types of injuries with diverse effects on the heart, can all converge into a common cardiac progenitor cell response mediated through a common stress-activated signaling pathway (Fernando and Megeney 2007, Abdul-Ghani and Megeney 2008).

Caspases, a family of cysteinyl-aspartate-specific proteases, are master regulators of apoptosis and their activation is considered mandatory to initiate and execute programmed cell death (Nicholson *et al.* 1995, Cohen 1997). However, recent findings suggest a number of non-apoptotic roles of caspases (Figure 4), like self-renewal and differentiation of ESCs (Fujita *et al.* 2008), hematopoietic stem cells (Janzen *et al.* 2008) as well as differentiation of skeletal muscle and neurons (Fernando *et al.* 2002, Fernando *et al.* 2005). Experimental activation of these proteases can be used for unraveling cell fate decisions beyond programmed cell death. For instance, transient caspase 3-activation is required for the differentiation of various cell types, including neural precursor cells, hematopoietic cells and skeletal myoblasts (Fernando *et al.* 2002, Fernando *et al.* 2005, Fernando and Megeney 2007, Janzen *et al.* 2008, Larsen *et al.* 2010). In mESC cells, inhibition of caspases 3 and 8 impairs cardiomyocyte differentiation (Hunter *et al.* 2007, Abdul-Ghani *et al.* 2011),

while doxycycline-mediated activation of caspase 3 stimulates differentiation and at the same time decreases the expression levels of Nanog and Oct4 (Fujita *et al.* 2008). A recent report by Akbari-Birgani et al. suggests that delay in the release of cytochrome C, apoptosome formation and caspase 3- activation discriminates apoptosis from differentiation in mouse ESCs (Akbari-Birgani *et al.* 2014). It has also been proposed that apoptosis and muscle differentiation induce similar cytoskeletal and phenotypical changes, for example actin fiber disassembly and reorganization, and thus share activation of similar molecular pathways (Fernando *et al.* 2002, Murray *et al.* 2008). A mechanism of action by which active caspase 3 induces cardiac differentiation might be through cleavage and activation of mitogen-activated protein kinase (MAPK) signaling cascades (MST1/MKK6/p38) (Fernando *et al.* 2002). Downstream components of these cascades up-regulate Mef2a and c factors, known to be important for cardiomyocyte differentiation (McKinsey *et al.* 2000). In the work by Abdul-Ghani et al. (Abdul-Ghani *et al.* 2011), the authors demonstrated that caspases mediate cardiac differentiation through their proteolytic cleavage of β-catenin, leading to a loss of canonical Wnt signaling activity (Steinhusen *et al.* 2000). Recently, Wang et al demonstrated that skeletal muscle dedifferentiation during newt limb regeneration depends on a programmed cell death response (Wang *et al.* 2015). The authors were able to derive a proliferating progeny from differentiated, multinucleated muscle cells by first inducing and subsequently intercepting a programmed cell death response (Wang *et al.* 2015). Since the dedifferentiation of existing, mature cardiomyocytes was proposed as a mechanism of heart regeneration in both zebrafish and neonatal mice (Jopling *et al.* 2010, Porrello *et al.* 2011), these findings suggest that manipulation of apoptotic mechanisms might be an attractive strategy to boost cardiac endogenous regenerative potential in response to injury.

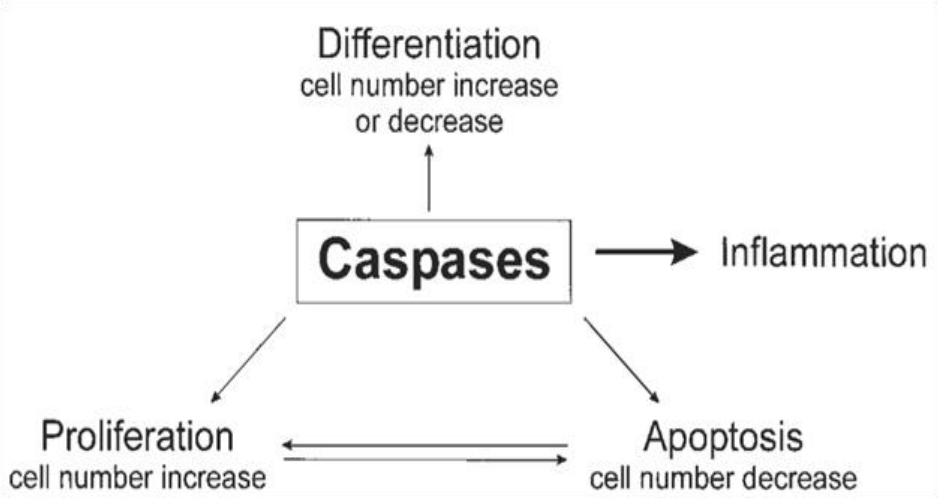

Figure 4. Emerging roles of mammalian caspases.

Caspases have been shown to play a role in cell proliferation, differentiation and apoptosis as well as cytokine processing during inflammation. Reprinted with the permission from the publisher of (Fadeel *et al.* 2000)

1.8 THE ROLE OF METABOLISM IN DEVELOPMENT AND CARDIOMYOCYTE DIFFERENTIATION

Rather than being only a consequence of the energy requirements of a cell, the metabolism is now also believed to play an active role in determining cell fate and regulating cell renewal and differentiation. This occurs through a shift in balance from glycolysis to oxidative phosphorylation (OxPhos) during maturation of stem cells, and vice versa during the reprogramming to pluripotency (Figure 5) (Folmes *et al.* 2011, Varum *et al.* 2011, Folmes and Terzic 2016). Consequently, the differentiation is characterized by cytosolic elongated and cristae-rich mitochondria, while reprogramming induces regression into a few spherical and cristae-poor perinuclear mitochondria (Folmes *et al.* 2011, Varum *et al.* 2011, Folmes and Terzic 2016). Studies of metabolism in various stem cell populations have identified tissue- and cell-specific metabolic pathways that are tightly regulated during development (Shyh-Chang *et al.* 2013, Takubo *et al.* 2013).

Wnt-signaling controls many biological processes including self-renewal and differentiation of cardiac progenitors. Moreover, regulation of the cellular metabolism may represent a general mechanism contributing to the wide-range of functions controlled by Wnt-proteins. For instance, it is suggested that Wnt3a induces transition

to aerobic glycolysis, known as the Warburg effect, by increasing the levels of key glycolytic enzymes (Esen *et al.* 2013). This metabolic regulation requires Lrp5 but not β-catenin, and is mediated by mTORC2-AKT signaling (Esen *et al.* 2013). A similar metabolic switch occurs in the earliest developmental stage from highly active mitochondria in naive (blastocyst ESCs) to highly glycolytic state in primed ESCs (in the post-implantation epiblast) (Figure 5) (Zhou *et al.* 2012, Takashima *et al.* 2014, Sperber *et al.* 2015). As development proceeds, the primed state, which mainly relies on glycolysis as energy source, rapidly changes to highly active OxPhos when the cells begin to differentiate.

Studies on undifferentiated adult BM-MCSs have shown that these cells rely on glycolysis for energy supply, while activating the mitochondrial processing through OxPhos during differentiation (Chen *et al.* 2008). A number of transcriptional and signaling pathways converge to regulate glycolysis, where the hypoxia-inducible factor (HIF) pathways are the best characterized (Papandreou *et al.* 2006, Goda and Kanai 2012, Semenza 2012). The metabolic switch from OxPhos to glycolysis occurs very early in the reprogramming process and requires both Hif1 and Hif2 proteins, each independently and stage-specifically (Mathieu *et al.* 2014, Prigione *et al.* 2014). The emerging role of hypoxia and the Hif in the acquisition of stemness is an example of metabolic context in cell fate (Zhou *et al.* 2012, Mathieu *et al.* 2013, Shyh-Chang *et al.* 2013, Mathieu *et al.* 2014, Shiraki *et al.* 2014). Through the stabilization of the Hif activity, stem cells acquire a metabolic profile (Zhou *et al.* 2012, Mathieu *et al.* 2014) that may be determinative for stemness (Zhou *et al.* 2012, Mathieu *et al.* 2014) and allows for a highly proliferative state. Indeed, hypoxia can induce the reversal of the early steps in human ESC differentiation (Mathieu *et al.* 2013).

However, the dependency of stem cells on glycolysis to produce ATP could also reflect an adaptation to low oxygen tensions *in vivo*, since hypoxia is a key feature of the stem cell niche (Suda *et al.* 2011). Glycolysis is optimal to fuel lower energetic demands of quiescent MSCs residing within niches with low oxygen, and limits oxidative metabolism-dependent generation of potentially damaging reactive oxygen species (ROS) (Mohyeldin *et al.* 2010, Suda *et al.* 2011, Ito and Suda 2014, Perales-Clemente *et al.* 2014). Besides BM-MSCs, several other tissue specific adult stem cells including long-term hematopoietic stem cells (Tothova *et al.* 2007) and neural stem cells (Renault *et al.* 2009) reside within *in vivo* niches with low oxygen tensions. These niches are suggested to maintain cells in a glycolytic quiescent state to ensure their long-term self-renewal by limiting the generation of ROS and subsequent ROS-induced cellular

damage (Ito and Suda 2014). Another consideration is that glycolysis is a preferred method of ATP production in proliferating cells and oxidative stress induced senescence has been shown to limit the expansion of MSCs in normoxic conditions.

1.9.1 **Cardiac metabolic transitions during differentiation and maturation**

An important cardiac regenerative strategy might be to deliver cardiogenic MSCs, where the studies of cellular metabolomics may provide knowledge about the cells that have the highest propensity to differentiate into functional cardiomyocytes. Cardiomyocytes undergo several changes in order to increase their metabolic capacity with age. One of the most significant metabolic changes in cardiomyocytes is the transition from anaerobic to oxidative metabolism (Sartiani *et al.* 2007).

In the fetal heart, cardiomyocyte progenitors preferably use lactate to fuel OxPhos (Werner and Sicard 1987). The fetal heart is adapted to a low oxygen environment and highly dependent on glycolysis to produce ATP (Lopaschuk and Jaswal 2010). Since lactate is plentiful in the fetal heart, fetal tissue will also produce a large part of its ATP via lactate oxidation.

After birth, the heart undergoes a period of massive cellular growth while adapting to an increased workload, and its energy demands cannot longer be satisfied via glucose and lactate oxidation alone (Lai *et al.* 2008). There is also a concomitant increase in circulating levels of free fatty acids that mediates a switch from predominant glycolysis in immature cardiomyocytes to predominant OxPhos as energy source in the postnatal heart (Kolwicz *et al.* 2013). As lactate levels fall during development, the cardiac tissue changes from being mainly dependent on lactate oxidation, to utilize fatty acids as primary fuel source (Kolwicz *et al.* 2013). Changes in metabolic substrates are coupled with mitochondrial reorganization in order to more efficiently power beating cardiomyocytes. As cardiomyocytes mature, the number of mitochondria in each cell increases and these mitochondria have highly developed cristae and are more densely packed than in young cardiomyocytes (Legato 1979). The adult cardiomyocytes need a high metabolic rate to fulfill a constant demand for ATP to power continual contractile activity (Schonfeld *et al.* 1996). Under normal physiological conditions cardiac ATP is predominantly generated by OxPhos using fatty acids as a substrate (Figure 5). However, mature cardiomyoctes have a very limited ability to store high energy phosphates (Lopaschuk and Jaswal 2010). A small amount of ATP is generated via

anaerobic glycolysis within the cytosol, and is of critical importance when mitochondrial function is limited (Taegtmeyer *et al.* 2004, Taegtmeyer *et al.* 2010).

This switch in metabolic substrate and reorganization of mitochondria are key components of cardiomyocyte development. When the respiratory chain function is experimentally disrupted during cardiac differentiation of stem cells, it compromises the ability of cardiomyocytes to reorganize their mitochondria and switch their metabolic profiles, resulting in impaired contractile machinery (Chung *et al.* 2007). It has also been suggested that the switch in metabolic profile is a potential regulator of cardiomyocyte maturation. Thus, unrevealing the mechanisms that contribute to changes in cardiac progenitors metabolic profile might lead to better understanding of cardiomyocyte maturation.

Recent work has identified a number of factors that regulate cardiomyocyte metabolic maturation, acting both inter- and intra-cellular. It was shown that the let-7 family of microRNAs works endogenously in cardiomyocytes to accelerate maturation, largely through a significant impact on cardiomyocyte metabolism (Kuppusamy *et al.* 2015). The let-7 miRNA family target genes are involved in the PI3/AKT/insulin pathway.

Recently, Puente et al. suggested that the increase in environmental oxygen and the subsequent up-regulation of oxidative metabolism is the upstream signal that triggers cell cycle exit of cardiomyocytes shortly after birth (Puente *et al.* 2014). To further address this hypothesis, Kimura et al. generated a transgenic mouse expressing a chimaeric protein in which the oxygen-dependent degradation (ODD) domain of Hif-1α is fused to the tamoxifen-inducible CreERT2 recombinase and were able to identify a rare population of hypoxic cardiomyocytes in the adult mice (Kimura *et al.* 2015). Interestingly, these hypoxic cardiomyocytes were smaller in size, mononucleated and displayed lower oxidative DNA damage, resembling the proliferative neonatal cardiomyocytes, and finally, contributed to new cardiomyocyte formation in the adult heart (Kimura *et al.* 2015).

Furthermore, Principal Component Analysis (PCA) on RNA-seq of human adult and fetal hearts, as well as on 20-day and 1-year old cardiomyocytes derived from H7 hESCs, showed that among the top genes that contributes to the separation of fetal versus adult samples there are many genes related to metabolism and OxPhos (Ellen Kreipke *et al.* 2016). Among the top metabolic genes, the glucose transporters (HEPH and SLC7A/SLC2A3) were mainly upregulated in the fetal but not the adult cardiomyocytes (Ellen Kreipke *et al.* 2016). Similarly, Nicotinamide N-Methyltransferase (NNMT) was upregulated in more mature cardiomyocytes compared

to fetal-like samples (Ellen Kreipke *et al.* 2016). While it was shown that NNMT represses Wnt and activates the HIF pathway in primed hESCs by controlling substrate availability for H3K27me3 histone methylation (Sperber *et al.* 2015), its role in cardiomyocyte maturation remains to be identified.

Interestingly, one of the hallmarks of pathological hypertrophy and heart failure is a re-expression of the fetal gene program. Common fetal genes that are upregulated include: atrial natriuretic factor (Anf), brain natriuretic peptide (BNP), and α-myosin heavy chain (αMHC). From a metabolic standpoint, there is also a reversion to a fetal-like metabolic state. However, it is not a complete reversion. As previously discussed, the fetal and neonatal heart rely on glycolysis as a source of energy, and thereby a related increased activity of glycolytic pathway associated enzymes (Fisher *et al.* 1980). Following birth, there is a 10-fold increase in fatty acid oxidation along with a decrease in glycolytic rates (Itoi and Lopaschuk 1993). In some forms of heart failure, this pattern is reversed (Allard *et al.* 1994, Nascimben *et al.* 2004) with either no change or a decrease in glucose oxidation (Leong *et al.* 2002, Kolwicz *et al.* 2013).

Post-ischemic myocardium is characterized by hypoxia, inflammation, and oxidative stress (Milano *et al.* 2004, Zhu *et al.* 2006, Garedew and Moncada 2008, Ong and Gustafsson 2012), all of which induce dysfunction of mitochondria, the gatekeepers of cell survival (Ott *et al.* 2007). The hypoxic environment of the myocardium activates HIF1, which, in turn, activates transcriptional programs to alleviate the effects of the low oxygenation, thus stimulating myocardial angiogenesis, cardiomyocyte growth, and reprogramming of metabolism (Semenza 2014). However, in the case of continued pathological stress, this compensatory mechanisms ability to rescue the hypoxic environment becomes detrimental.

Characterization of the metabolism of MSCs under different oxygenation conditions would, thus, provide critical insight for identifying pathways important for reducing cell vulnerability to the hostile post-ischemic myocardial microenvironment (Forrester *et al.* 2009, Mohsin *et al.* 2011) and subsequent optimization of functional engraftment. Finally, metabolic flexibility of a quiescent stem cells might be critical for endogenous regeneration pointing to oxygen as a major factor for regulation of tissue repair.

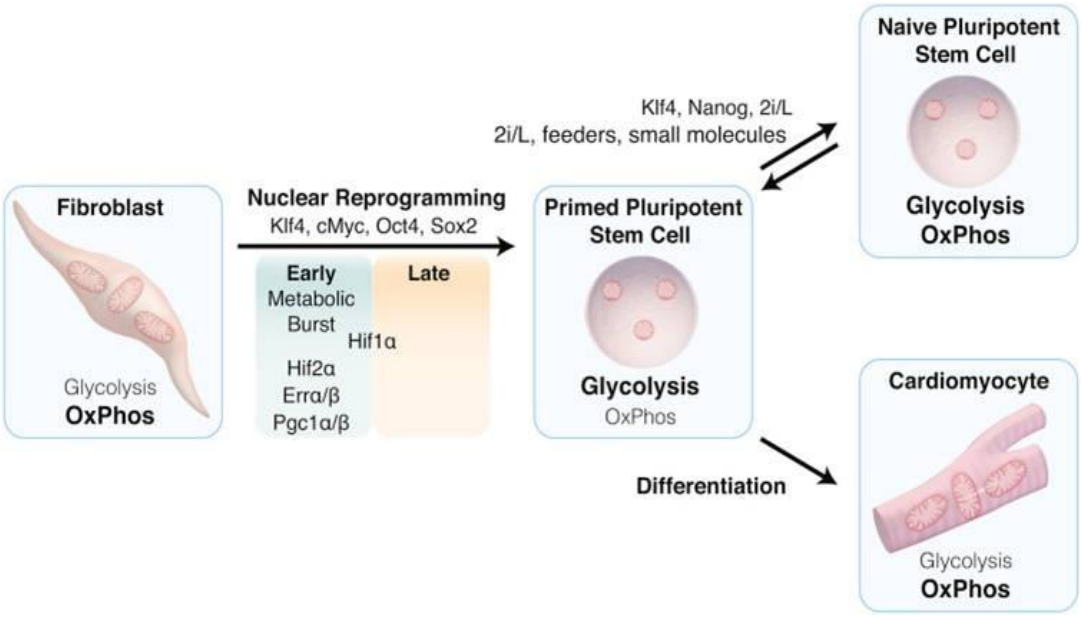

Figure 5. Global metabolic changes in cellular fate transitions

Transition between the naive and primed pluripotent states in human cells is associated with changes in the metabolism from predominantly glycolytic to bivalent metabolism utilizing both glycolysis and oxidative metabolism. In contrast, upon a differentiation stimulus, pluripotent stem cells activate mitochondria biogenesis to generate a robust network of mitochondria in support of oxidative metabolism to match lineage-specific energetic demands. Reprinted with the permission from the publisher of (Folmes and Terzic 2016) http://dx.doi.org/10.1016/j.semcdb.2016.02.010

1.9 CELL-BASED THERAPIES IN HEART FAILURE

Heart failure, most often subsequent to ischemic heart disease, is a major cause of morbidity and mortality worldwide (Roger 2013). After a large myocardial infarction, more than a billion cardiomyocytes are lost (Laflamme and Murry 2005), and although substantial advances have been made in medical treatment improving the prognosis of these patients (Birks *et al.* 2006), none of the current therapeutic approaches directly targets the loss of cardiomyocytes, which is the underlying cause of heart failure.

For decades, the heart was viewed as a terminally differentiated organ lacking the regenerative capacity sufficient to replace the loss of myocytes following injury. The discovery of endogenous cardiac progenitor cells and reports demonstrating a low turnover of existing cardiomyocytes have changed this view to some extent (Hierlihy *et al.* 2002, Beltrami *et al.* 2003, Bergmann *et al.* 2009, Porrello *et al.* 2011,

Bergmann *et al.* 2015). Whether these new cardiomyocytes are derived from the proliferation of existing cardiomyocytes or from cardiac progenitor cells is still under discussion.

Multiple candidate cell types have been used in preclinical animal models and in humans to repair or regenerate the injured heart either directly or indirectly (through paracrine effects). Although no consensus has emerged, the ideal cell type for treatment of heart disease should: be safe i.e. not generate tumors nor induce arrhythmias; improve heart function; integrate to the host tissue; improve the functional capacity of the surrounding cardiomyocytes; stimulate angiogenesis; be delivered by minimally-invasive clinical methods; be tolerated by the immune system and be ethically acceptable (Madonna *et al.* 2016).

In the early 1990s the first skeletal myoblasts were isolated, expanded and successfully transplanted to an injured heart opening the door for the cardiac cell therapy (Figure 6) (Chiu *et al.* 1995). Since this pioneering work several animal studies have provided evidence for the possibility of intra-myocardial myoblast grafting (Taylor *et al.* 1998, Dowell *et al.* 2003, He *et al.* 2005, van den Bos *et al.* 2005). In the first clinical phase I trials these cells were implanted concomitantly to coronary artery bypass grafting (CABG) (Menasche *et al.* 2001, Hagege *et al.* 2003). This study was followed by three other adjuncts to CABG studies, reporting similar positive effects on the myocardial function (Dib *et al.* 2005, Gavira *et al.* 2006). In sharp contrast to the functional benefit observed in the early-uncontrolled studies, the first prospective randomized placebo-controlled phase II trial (MAGIC trial), reported lack of efficacy as well as increased early postoperative arrhythmic events (Menasche *et al.* 2008), thus ending the use of myoblasts for heart regeneration.

Another set of cells that fuelled the field of cardiac regeneration was somatic stem cells, among which, the most widely studied are the bone marrow-derived hematopoietic stem cells. The study that first explored the potential of hematopoietic stem cells in heart regeneration was the study by Orlic and co-workers from 2001(Orlic *et al.* 2001), where they demonstrated that Lin-/ c-Kit+ cells could regenerate 68% of the infarcted area in a mouse model. These findings could not be repeated by other groups (Balsam *et al.* 2004, Murry *et al.* 2004) and according to the results by Nygren and co-workers part of the effect exerted by these stem cells might be due to cell-fusion (Nygren *et al.* 2004). The relative accessibility of bone marrow, the large numbers of unfractionated autologous cells that can be obtained without ex vivo expansion and the extensive experience with bone marrow transplantation facilitated clinical application. The BM-

MNCs used in these clinical trials (Wollert *et al.* 2004, Schachinger *et al.* 2006, Assmus *et al.* 2010) were mainly isolated by density centrifugation and the vast majority of the cells were committed hematopoietic cells at various stages of maturation (Dimmeler and Zeiher 2009). These first generation cell therapies have achieved only minor effects (Gyongyosi *et al.* 2015), possibly attributed to paracrine mechanisms without the evidence of replacement of lost cardiomyocytes (Garbern and Lee 2013). In a recent meta-analysis of 31 randomized cell therapy trials in heart failure, which included 1521 patients, exercise capacity, left ventricular ejection fraction (EF), and quality of life were found to be improved in the treated patients (Fisher *et al.* 2015). However, when only double-blind studies were selected, the difference was no longer significant (Assmus *et al.* 2015), (Fisher *et al.* 2015). The effect of BM-MNC therapy on mortality after acute myocardial infarction (AMI) will be evaluated in the ongoing phase III BAMI trial (NCT01569178).

The initial trial with c-Kit+ progenitor cells grown from an intra-operatively harvested right appendage biopsy (SCIPIO) had controversial results (Bolli *et al.* 2011).

However, the studies that have compared the effect on cardiac function between different cell types, have consistently demonstrated superiority of cardiac-committed cells (c-Kit+ or Sca1+ cardiac stem cells, cardiospheres, iPSC-derived cardiomyocytes) over cells not committed to a cardiac lineage such as BM-MNCs, MSCs or skeletal myoblasts (Matsuura *et al.* 2009, Rossini *et al.* 2011, Li *et al.* 2012). Thus, the next generation cell therapies make use of alternative strategies, based upon activation of molecular pathways and transcription factors guiding cardiac differentiation (Figure 6). Lineage-specified cardiopoietic MSCs, cardiomyocytes derived from embryonic stem (ES) cells, or heart fibroblasts directly converted into cardiomyocytes by inducing core cardiac transcription factors are being used for cardiac repair in experimental models as in clinical trials (Ieda *et al.* 2010, Song *et al.* 2012, Bartunek *et al.* 2013, Chong *et al.* 2014, Menasche *et al.* 2015). By applying similar signals that instruct precardiac mesoderm to commit into the cardiomyogenic fate during heart development (Behfar *et al.* 2008), BM-MSCs have been induced to activate a cardiovascular lineage-specifying program (Behfar *et al.* 2010). The first clinically tested example of that approach is tested in the C-Cure trial of patients with ischemic cardiomyopathy (Bartunek *et al.* 2013). The positive results of the initial C-CURE study (Bartunek *et al.* 2013) were not, however, reproduced in the European phase III trial Congestive Heart Failure Cardiopoietic Regenerative Therapy (CHART-1), which had as a primary efficacy

endpoint a composite of mortality, worsening of heart failure, 6-min walk test, left ventricular end-systolic volume, and left ventricular EF at 9 months (Bartunek *et al.* 2016).

Another class of the second-generation cells is the PSCs, both ESCs and iPSCs (Figure 6). A prerequisite for clinical application of PSCs for heart repair is their efficient and strict differentiation into cardiomyocytes and endothelial cells, without risk for side effects (Menasche and Vanneaux 2016). The ESCORT trial, a clinical trial where ESC-derived Ssea-1+ Isl1+ early mesodermal/cardiac progenitor cells embedded in a fibrin scaffold are applied concomitantly with a CABG operation, has been initiated in France (Menasche and Vanneaux 2016). The primary end point of this pilot phase 1 trial is safety with a particular focus on tumor formation and on development of ventricular arrhythmias, since ventricular arrhythmias have been reported in the non-human primate study (Chong *et al.* 2014). iPSC-derived cardiomyocytes have not yet been tested in humans, largely because of the increased risk of genetic mutation inherent to the reprogramming method as such (Menasche *et al.* 2015). Although PSCs derived cardiac progenitors and direct conversion of cardiac fibroblasts into cardiomyocytes hold a great regenerative promise, the cardiomyocytes generated by these strategies still possess the immature features of embryonic cardiomyocytes (Snir *et al.* 2003, McDevitt *et al.* 2005, Abdul Kadir *et al.* 2009, Lieu *et al.* 2009). Future studies are needed to optimize the differentiation protocols in order to make these cells more similar to mature ventricular cardiomyocytes and thus reduce the risk of arrhythmias (Lambers and Kume 2016).

Further optimization of cardiac stem cell therapies is needed to increase the pool of cardiomyocytes to the level that prevents or reverses the negative remodeling of the myocardium in order to prevent heart failure. Consequently, multiple strategies have been developed to empower stem cells to survive in the hostile environment through pharmacological, genetic or preconditioning interventions (Mohsin *et al.* 2011). Importantly, most studies report a sharp discrepancy between minimal cell engraftment of transplanted cells and their putative beneficial effect on preservation of left ventricular function (den Haan *et al.* 2012, Menasche and Vanneaux 2016). Yet, the mechanisms of action are only starting to be unraveled. It has been demonstrated that cells secrete nano-sized extracellular vesicles (ECV), which can transport and transfer microRNAs, proteins, lipids and genetic material, thus

activating endogenous cytoprotective pathways (Cheng *et al.* 2014, Bobis-Wozowicz *et al.* 2015, Zhang *et al.* 2016).

In a study by Tseliou et al, cardiosphere-derived ECVs altered the phenotype of fibroblasts enabling them to engage in angiogenesis and cardioprotection (Tseliou *et al.* 2015). A precise characterization of cell-released factors accounting for the beneficial effects of ECVs remains to be elucidated. Furthermore, there is a need to evaluate whether the effect of ECVs on cardiac repair is relevant in a clinical setting and in addition, offer major advantages in regard to facilitating the manufacturing process as well as circumventing regulatory hurdles that are linked to cell-based therapies (Menasche and Vanneaux 2016, Zhang *et al.* 2016).

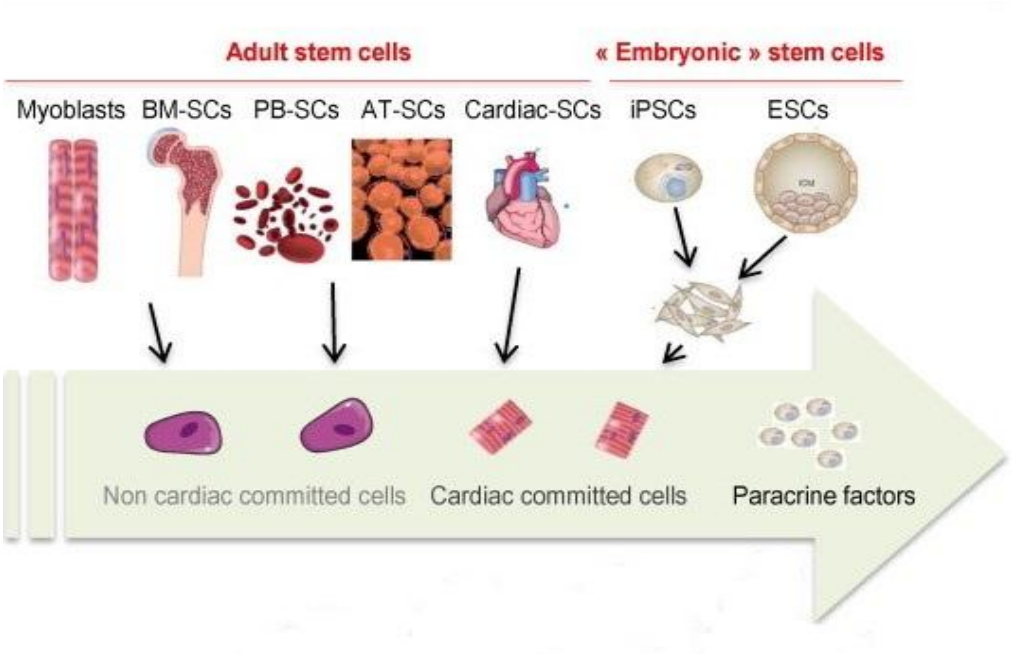

Figure 6. The evolution of the stem cell therapy for heart failure.

Initially, the majority of trials used adult stem cells as bone marrow- derived or peripheral blood-derived MNCs. However, the best outcomes were achieved using therapeutic cells with cardiac progenitor characteristics, leading to development of cardiac-committed cell therapies.
Recent findings are profoundly changing regenerative treatment paradigm by suggesting that therapeutic cells act as reservoirs of paracrine factors that may trigger endogenous repair potential. Abbreviations: BM: bone marrow, PB: peripheral blood; AT: adipose tissue; iPSCs: induced pluripotent stem cells; ESCs: embryonic stem cells; SCs: stem cells. Adapted from (Silvestre and Menasche 2015) http://dx.doi.org/10.1016/j.ebiom.2015.11.010

A part of this introduction chapter is adopted from our review article with the permission of the publisher (Bulatovic *et al.* 2016).

2 AIMS OF THE THESIS

The overall aims of this thesis were to explore the potential of human fetal cardiac MSCs to be used for cardiac regeneration and if sublethal apoptotic stimuli and metabolic cues could influence cardiomyocyte progenitors plasticity in terms of proliferation and differentiation ultimately leading to boost the endogenous regenerative potential in injured hearts.

The specific aims of each publication are listed as follows:

Study I: to explore if apoptotic stress in terms of sublethal caspase activation influences the proliferation and differentiation of cardiac progenitor cells

Study II: to develop a defined and reproducible protocol for large-scale expansion of human fetal cardiac MSCs with cardiac progenitor characteristics to be used for generation of endothelial cells, smooth muscle and cardiomyocytes

Study III: to characterize the metabolomic profile of MSCs derived from human fetal hearts of the first trimester

3 MATERIALS AND METHODS

This chapter contains a detailed, comprehensive description of the most relevant methods applied in the studies I-III. The additional laboratory techniques are described in the respective papers.

3.1 CELL CULTURE AND DIFFERENTIATION - STUDIES I-III

Mouse Embryonic Stem Cells - Study I

Murine embryonic stem cells (mESC-line CGR8) were kindly provided by Prof. Richard Lee, Harvard Medical School. The mESCs were stably transfected with the cardiac-specific α-myosin heavy chain (α-MHC) promoter tagged with green fluorescent protein (GFP) (Chan *et al.* 2009). Upon differentiation into cardiomyocytes, these cells express GFP, which could be quantified and monitored in fixed cells as well as in live cultures. The cells were propagated in Glasgow Minimum Essential Medium (GMEM) (Invitrogen) supplemented with 1,000 U/ml Leukemia Inhibitory Factor (LIF, ESGRO, Millipore), 1mM Sodium Pyruvate, 1x Non-Essential Amino Acid, 15% Knockout Serum Replacement (Invitrogen), 10-4Mβ-mercaptoethanol, and 1 x Penicillin-Streptomycin. Differentiation was carried out as hanging drops in differentiation medium (10% FBS without LIF) in which embryoid bodies (EBs) were formed in 2 days (D0–2). For caspase activation experiments EBs were cultured in differentiation medium for three weeks, then incubated for 5 hr with 100 nM STS followed by a recovery period ranging from 24 hr to 7 days.

Derivation and Expansion of Human Fetal Cardiac MSCs - Study II and III

The fetal hearts were obtained from legal terminations of pregnancy after the donor's informed consent and ethics approval from the Regional Ethics Board in Stockholm.
The fetal hearts were pre-digested overnight at 4 C in a 0.5 mg/ml Trypsin solution in Hank's balanced salt solution (HBSS). The MSC fraction was prepared according to a modified version of the protocol developed by (Laugwitz *et al.* 2005). The pre-

digested heart was treated for 15 min with collagenase type II (Worthington Biochemical) 160 U/ml in HBSS, at 37 C under gentle stirring. The supernatant was centrifuged at 220 g for 8 min and the pellet resuspended in low-glucose DMEM supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories), Mycozap (Lonza, Switzerland) and GlutaMAX (Invitrogen). The digestion with collagenase was repeated until the tissue was completely dissociated. The pooled cells were washed twice and resuspended in the same medium as above. The cells were subsequently seeded on plastic and cultured for 96 hr in order to recover the adherent fraction and thereafter cryopreserved for later use. The adherent cell fraction obtained with this method contains a mixture of different cell types, including the cardiac MSCs.

To activate the transcriptional network known to be involved in embryonic cardiogenesis, we focused on the canonical Wnt/β-catenin signaling pathway, which has previously been shown to be effective in expansion of SHF progenitors derived from embryonic stem cells from mice and humans (Moretti *et al.* 2006, Qyang *et al.* 2007, Bu *et al.* 2009).

Based on a strategy previously introduced by Qyang et al., we used a Wnt3a-containing cell culture medium (Qyang *et al.* 2007). When cells of the adherent cell fraction of human fetal hearts were expanded on plastic, they multiplied rapidly but showed a low expression of the cardiac progenitor markers Isl1, Kdr, and Nkx2.5, as well as the pluripotency marker Oct4 (Mansson-Broberg *et al.* 2016). In order to improve the culturing conditions, the adherent cells were cultured on dishes pre-coated with the Engelbreth-Holm Swarm mouse tumor-derived extracellular matrix extract, Geltrex (GibcoBRL). This extract, which contains basement membrane proteins such as LN-111, has been widely used for culturing stem cells and other cell types (Xu et al., 2001; Ludwig et al., 2006). When the adherent cells were cultured on Geltrex in combination with Wnt3A-containing medium, the cells divided exponentially and were passaged every other day. The cells were seeded at a density of 30 000 cells/cm2 on cell culture plastic coated with a thin layer of Geltrex in DMEM/F12 supplemented with B27 (GibcoBRL), 2% FBS (PAA), MycoZap (Lonza), epidermal growth factor (10 ng/ml) (R&D Systems) and 100 ng/ml recombinant human Wnt3A (R&D Systems). In order to validate the cell culture protocol, different culturing conditions were tested, where either Geltrex or Wnt3A was excluded (gestational weeks 6.5–8, n = 3). After each passage, cells were saved for further analyses and at the end of the experiment, at 2 and 3 weeks, the cells were

harvested for immunocytochemistry, PCR, and microarray analyses. Exclusion of either Wnt3A or Geltrex from the culture protocol negatively affected proliferation of the cells in the initial fraction and preserved survival of the limited numbers of remaining cardiomyocytes among the adherent cells. These data suggest that both the Wnt3A-containing medium and Geltrex may provide important signals for expansion of cells from the adherent cell fraction of fetal human hearts, with transcriptional signatures akin to those of cardiac progenitors (Mansson-Broberg *et al.* 2016).

Laminin-Based Derivation and Expansion of Human Fetal Cardiac MSCs - Study II

Cultured pluripotent stem cells and pluripotent cells of the inner cell mass of the blastocyst express LN-511 and LN-521 (Rodin *et al.* 2010). Since these LNs have previously been demonstrated to support clonal expansion of cultured embryonic stem cells (Rodin *et al.* 2014) and the LN subunits α5 and γ1 are also abundantly expressed in human fetal hearts, this suggests that LN-511 and LN-521 may be suitable for derivation and expansion of cardiac MSCs. LN-211, on the other hand, might be linked to cardiomyocyte development and/or function, as it has been shown to be defective in congenital muscular dystrophic patients with cardiomyopathy (Finsterer *et al.* 2010), and α2 chains were also expressed in the fetal heart.

To generate chemically defined matrices for derivation and expansion of human fetal cardiac MSCs (gestational weeks 6.5–8), the cells were grown in wells coated with LN-511, 521, or 211, using the same culture medium as described above. The human recombinant laminins were generated as previously described (Kortesmaa *et al.* 2000, Doi *et al.* 2002, Rodin *et al.* 2014) or purchased from BioLamina AB (batches 80,051, 80,050, and 80,042, respectively).

MSCs cultured on LN-511 and LN-521 displayed a tendency toward increased mRNA levels of Isl1 in comparison with cells cultured on Geltrex. For cardiac differentiation fetal cardiac MSCs derived and expanded on LN-521 were cultured on LN-211 in a medium devoid of Wnt3a.

Differentiation of Cardiac MSCs into Cardiomyocytes, Smooth Muscle Cells, and Endothelial Cells - Study II

For cardiomyocyte differentiation in vitro we used a modified protocol developed for human pluripotent stem cells (Lian *et al.* 2012), where the cardiac MSCs were cultured on Matrigel, LN-211 or plastic. Cardiac MSCs derived from fetal hearts of different ages (gestational week 6, 8, and 9, n = 3) were used. Canonical Wnt signaling was blocked using a defined serum-free medium to induce cardiomyocyte differentiation. After 3 weeks, cells were fixed in 4% phosphate-buffered formalin, stained with a mouse monoclonal antibody against human troponin T (TnT) (ab8295, clone 1C11, Abcam) and visualized with an Alexa Fluor 488-conjugated rabbit anti-mouse secondary antibody (A11059, Thermo Fisher Scientific). RNA extraction and qRT-PCR for TNNT2 and GAPDH were performed. For imaging calcium signaling dynamics, cells were labeled with the Ca2+-sensitive fluorescence indicator Fluo-4/AM (10 mM; Molecular Probes, Thermo Fischer Scientific) according to the manufacturers' protocol.

For differentiation toward vascular smooth muscle cells, the cardiac MSCs were seeded on gelatin-coated plastic, and cultured for 18 days in a defined medium supplemented with PDGF-BB (10 ng/ml) and TGF-β1 (2 ng/ml) (PeproTech), according to a modified protocol previously described by (Cheung *et al.* 2014). Cells were passaged 1:4 once per week into new gelatin-coated wells. Subsequently, the cells were fixed and stained with a mouse antihuman a-smooth muscle actin primary antibody (A2547, clone 1A4; Sigma-Aldrich) and visualized by a rabbit anti-mouse Alexa Fluor 488-conjugated secondary antibody (Thermo Fischer Scientific). The experiment was performed in duplicate using cells from four different hearts of gestational age 8 and 9 weeks.

Endothelial cell differentiation was induced in fetal cardiac MSCs by using a medium containing vascular endothelial growth factor (VEGF). The substrates used were gelatin or LNs present in the basal membranes of blood vessels (LN-521 and LN-411) (Hallmann *et al.* 2005, Domogatskaya *et al.* 2012). Fetal cardiac MSCs were seeded in culture wells pre-coated overnight with a 1:4 mixture of LN-521/ 411 or gelatin (0.1%) and cultured for 21 days in EGM-2 medium (Lonza) with VEGF (50 ng/ml) from R&D Systems (Liu *et al.* 2007). Subsequently, the cells were fixed and stained with a monoclonal mouse anti-human CD31 antibody (M0823, clone JC70A). Stained cells were visualized using an Alexa Fluor 568-conjugated goat anti-mouse

secondary antibody (Thermo Fischer Scientific). The experiment was performed in duplicate using cells from five different hearts of gestational age 8 and 9 weeks.

3.2 CONTINUOUS VISUALIZATION OF LIVE CELLS - STUDY I

Prospective visualization of GFP expression levels was done using CELL-IQ SLF continuous live cell imaging and analysis system platform (CM Technologies, Tampere, Finland). EBs growing in 24-well dishes were stimulated as indicated and then placed into the CELL-IQ imaging system. During the imaging period, conditions were maintained at 37°C, 5% CO2. A whole-well fluorescence confocal z-stack composed of 6x6 images was taken for each well for every 60 minutes, during a total recording time of 72 h. The culture medium was replaced every second day. Single cell tracking analysis was performed off-line using CELL-IQ analysis software.

3.3 MITOCHONDRIAL MORPHOLOGY ASSESSMENT - STUDY III

In addition to bioenergetic profiling, which reflects the amount of energy being produced by mitochondrial versus non-mitochondrial respiration, we assessed another dynamic feature that responds to the metabolic needs of the cell, the mitochondrial morphology. Presentation of mitochondria as separate organelles is usually associated with low activity while elongation and fusion into a network indicates activation and increased oxidative phosphorylation (Brand and Nicholls 2011). To visualize mitochondria, cells were incubated with MitoTracker Red (500 nM) and Green (200nM) (Invitrogen) for 1 hour, following which cells were fixed and visualized with confocal microscopy. Electron microscopy (EM) was performed as previously described (Folmes *et al.* 2013).

3.4 BIOENERGETIC AND METABOLOMIC PROFILING - STUDY III

Live cell XF24 bioenergetic profiling

XF24 Extracellular Flux Analyzer (Seahorse Biosciences) allows simultaneous assessment of two major cellular energy producing pathways, glycolysis and mitochondrial respiration by continuous real-time measurement of pH and oxygen concentration in live cells. This automated system measures the extracellular acidification rate (ECAR) of the assay medium (lactate, a byproduct of glycolysis, is

the major contributor of protons) as a surrogate of glycolytic flux and the oxygen consumption rate (OCR), which represents mitochondrial respiration.

Seahorse XF24 cell culture plates were coated with Geltrex TM (GibcoBRL) over night at 4°C, followed by 2 h at 37°C and cells were plated into wells of a XF24 microplate and maintained overnight to ensure 80% confluence. For the experiments, 30-50 000 cells were seeded in each well. Each microplate contained four empty wells that served as temperature controls. Prior to assay, plates were equilibrated in the absence of CO_2 for 1 h in unbuffered XF assay medium supplemented with 25 mM glucose, 2 mM glutamax, 1 mM sodium pyruvate, 1x nonessential amino acids and 1% FBS.

The Seahorse XF24 uses modulators of respiration that target components of the electron transport chain (ETC) in the mitochondria to measure key parameters of metabolic function. Oligomycin inhibits ATP synthase (complex V) and the decrease in OCR following injection of oligomycin correlates to the mitochondrial respiration associated with cellular ATP production. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential. As a result, electron flow through the ETC is uninhibited and oxygen is maximally consumed by complex IV. The FCCP-stimulated OCR can then be used to calculate respiratory capacity, defined as the difference between maximal respiration and basal respiration. Respiratory capacity is a measure of the ability of the cell to respond to increased energy demand. The third injection is a mix of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor. This combination shuts down mitochondrial respiration and enables the calculation of nonmitochondrial respiration driven by processes outside the mitochondria. In our experiments mitochondrial processes were interrogated by serial addition of oligomycin (0.5 μg/ml), FCCP (1 μM) rotenone (Rot, 0.5 μM) and antimycin (AntA, 1mM). The following OxPhos indexes were calculated: basal respiration (OCRpre-Olig - OCRpost-AntA), ATP-linked respiration (OCRpre-Olig - OCRpost-Olig), maximal respiration (OCRpost-FCCP - OCRpost-AntA) and respiratory capacity (OCRpost-FCCP - OCRpre-Olig). Each plotted value is the mean of 6 to 10 replicate wells, and is normalized to total protein quantified using a Bradford protein assay (Bio-rad). All data are presented as mean±SD from three separate experiments performed. Statistical differences were calculated using the Student's t test and considered significant at $*p<0.05$.

Cell preparation for nuclear magnetic resonance spectroscopy (NMR)

After harvesting, cell extraction was performed on 1-10×10^6cells per sample. 500 µl of a 2:1 (v/v) ratio of ice-cold methanol/chloroform was added to the frozen cell pellet, and the pellet–solvent mixture was sonicated for 4 min. Then, 500 µl of ice-cold1:1(v/v) chloroform/water was added and mixed to form an emulsion. After approximately 15 min, the samples were centrifuged at 13 000 rpm for 20 min. The upper phase (methanol and water) and lower phase (organic) were removed and placed in separate Eppendorf tubes taking care not to disturb the pelleted debris. The solvent was dried and the tubes were placed in a freezer until analyzed. For the NMR experiment, the lyophilized extracts were dissolved in 0.1 M phosphate buffer (pH 7.0) and 10% v/v D2O contained DSS (4, 4-dimethyl-4-silapentane-1-sulfonic acid), which is a chemical compound used in proton- and carbon-related NMR spectroscopy, as internal standard. Three samples per group were prepared in 5-mm NMR tubes (Optima Inc., Elk Grove Village, IL, USA). NMR spectra were recorded on a Bruker 500 MHz NMR spectrometer using noesypr1d pulse sequence with an 11160.7 Hz spectral width, 32k points, acquisition time of 1.4680 s, relaxation delay of 14 s and 64 scans. Spectra were processed with exponential line broadening to 0.3 Hz, zero filled to 64k points, and manually phase corrected using Topspin 3.1 prior to submission to Chenomx software for NMR data analysis.

Metabolomic footprinting

Extracellular metabolites ("metabolomic footprint") were quantified using proton nuclear magnetic resonance spectroscopy (1H NMR)(Folmes *et al.* 2011). Media samples were serially collected at 24 h. Media (540µL) was then added to 60 µL of D2O (Sigma) containing 5 mM sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 (TSP) (Sigma) for chemical shift reference and 81.84 mM formate (Sigma) for peak quantification reference. p-Toluene sulfonic acid (Sigma) was utilized as a reference standard to calibrate the formate concentration for quantitative analysis. Samples were filtered through Costar Spin-X filters and added to 5 mm NMR tubes (WilmadLabglass) for 1H NMR analysis on a BrukerUltrashield 700 MHz spectrometer using a zgpr water pre-saturation pulse with an 11160.7 Hz spectral width, 32,000 points, acquisition time of 1.4680 s, relaxation delay of 14 s and 64 scans. Spectra were processed with exponential line broadening to 0.3 Hz and zero filling to

65,000 points. Following Fourier transformation, spectra were autophased with phase correction, baseline corrected using a Bernstein polynomial fit and referenced to the TSP peak (0.00 ppm) using MestReNova 5.3.2 (MestRelab Research). Metabolite identities were assigned by comparison to reference values for chemical shift and multiplicity, and confirmed by comparison to spectra of pure compounds in the Human Metabolome database (Govindaraju *et al.* 2000, Wishart *et al.* 2009). Net fluxes of metabolites were calculated by subtracting normalized concentrations of metabolites in basal media from concentrations of metabolites in 24-h conditioned media. Rates were normalized to total cellular protein content determined by a Bradford protein assay (Bio-rad).

Spectral analysis and statistical analysis

The NMR data sets were referenced to DSS signal and baseline-corrected in the ChenomxNMR Suite 5.1 (Chenomx Inc., Edmonton, AB, Canada). Metabolite concentrations were measured using the 500 MHz library from ChenomxNMR Suite version 5.1, which uses the concentration of a known reference signal (DSS) to determine the concentration of individual compounds. The library is based on a database of individual metabolite spectra acquired using the NOESYPRESAT sequence. For multivariate statistical analysis spectra were binned (equidistant 0.04 ppm binning) with total peak area normalization and solvent region removal (4.68-5.00 ppm). Orthogonal projection to latent structures discriminant analysis (OPLS-DA) was carried out to discriminate between groups using SIMCA-P (version 11, Umetric, Umea, Sweden). The OPLS model maximizes the covariance between the measured data of X variable (peak intensities in NMR spectra) and the response of Y variable (classification components) within the groups. All data are presented as mean±SD from three separate experiments performed. Statistical differences were calculated using the Student's t test and considered significant at $*p<0.05$.

4 SUMMARY OF RESULTS

4.1 **STUDY I**

In response to apoptotic stress the adult heart reactivates gene expression programs normally found in the prenatal heart, which, in turn, is thought to allow protective changes of cell survival mechanisms, metabolism and contractility (Rajabi *et al.* 2007). Additionally, the dedifferentiation of adult cardiomyocytes was proposed as a mechanism of heart regeneration in zebrafish and neonatal mice (Jopling *et al.* 2010, Porrello *et al.* 2011). Based on these findings, in the Study I we initially hypothesized that proliferating progenitors could be derived from mature cardiomyocytes by first inducing and subsequently intercepting a programmed cell death response. However, finding the optimal model to explore this hypothesis *in vitro* has proved to be a challenge since adult cardiomyocytes were shown to spontaneously dedifferentiate in culture (Zhang *et al.* 2010). Therefore, we chose to optimize the sublethal apoptosis induction protocol on mouse ESCs that have been differentiated for three weeks, since, after that time point, most of the beating cardiomyocytes had stopped proliferating.

Treatment with nano-molar concentrations of the broad-spectrum apoptotic agent, Staurosporine (STS), induced activation of caspase-3 and -9, but failed to promote cell death. Sublethal caspase activation led to increased yield of cardiomyocytes, detected as increase of GFP expression, which was under control of the α-myosin heavy chain (α-MHC) promoter and up-regulation of mRNAs levels of specific cardiomyocyte markers (Figure 7). The broad-spectrum caspase inhibitor Q-VD-OPH abolished these effects. Interestingly, beating clusters of cells with low GFP expression were also observed following STS exposure, but not in the untreated control cells (Figure 7). Further investigation using single cell tracking is required to understand with certainty whether these early cardiomyocytes with low α-MHC expression were generated from cardiac progenitor cells or dedifferentiated cardiomyocytes.

Additionally, in our study, a broad caspase inhibition prevented up-regulation of cardiac-specific markers during EB-differentiation, similarly to what has been described for skeletal muscle differentiation (Murray *et al.* 2008) (Figure 8).

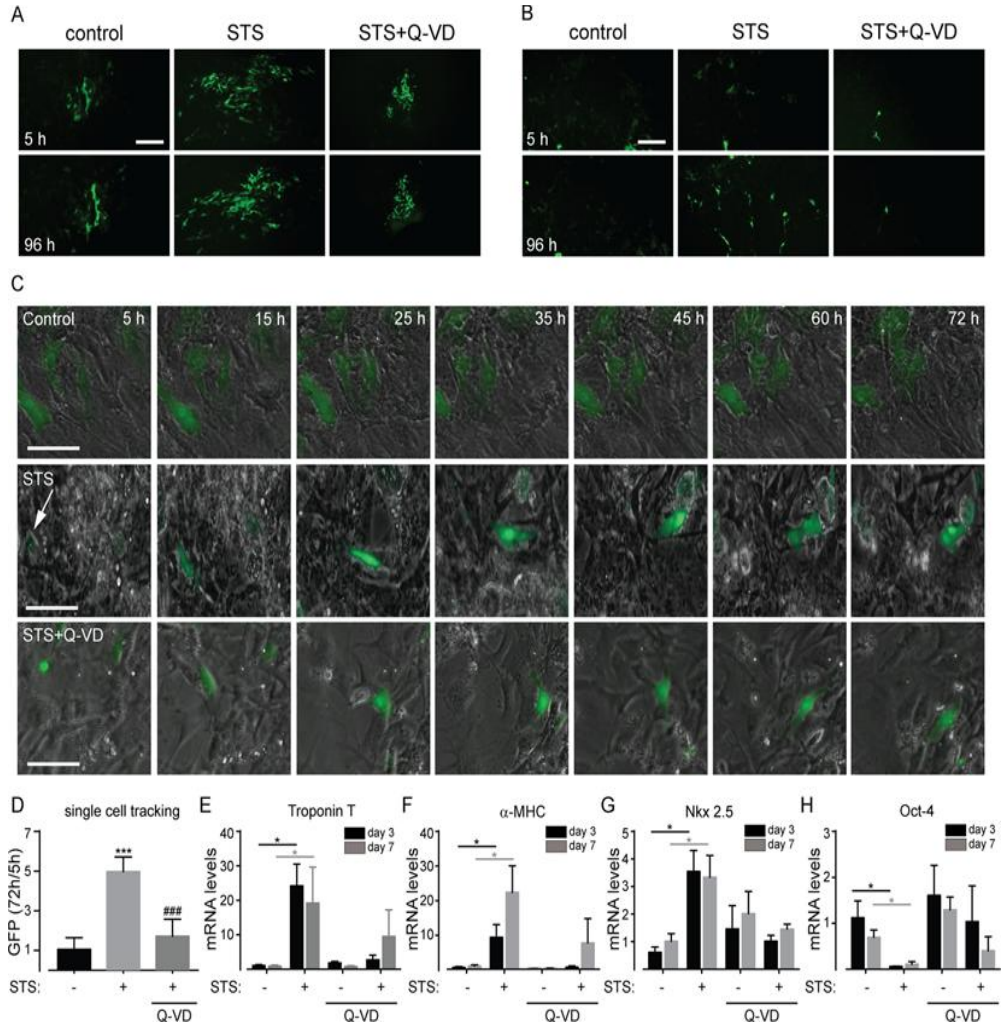

Figure 7. Cardiac differentiation is enhanced in response to sublethal proapoptotic stimuli

Mouse ESCs, stably transfected with a GFP reporter under the control of the α-MHC promoter were cultured as embryonic bodies in differentiation medium for three weeks followed by 5 h incubation with 100 nM STS. After a 96 h recovery, we observed an increase in the proportion of GFP+ cells in STS treated EBs. Beating clusters of cells with low GFP expression were also observed following STS exposure, but not in the untreated control cells (A). Pre-incubation of EBs with a broad- spectrum caspase inhibitor, Q-VD-OPH prevented the effects induced by STS (A and B). Subsequently, we continuously monitored GFP levels for 3 days after STS treatment using long-term live cell imaging. In control cells, GFP levels remained unaltered, while STS treatment significantly increased the GFP levels in single cells, which is under control of the α-MHC promoter, suggesting increased differentiation of early cardiomyocytes (C and D). TnT (E), α-MHC (F), and Nkx2.5 (G) mRNA expression levels were significantly up-regulated in STS-treated cells after 3 and 7 days of culture, whereas the expression of Oct4 (H), a hallmark of pluripotency, was downregulated.

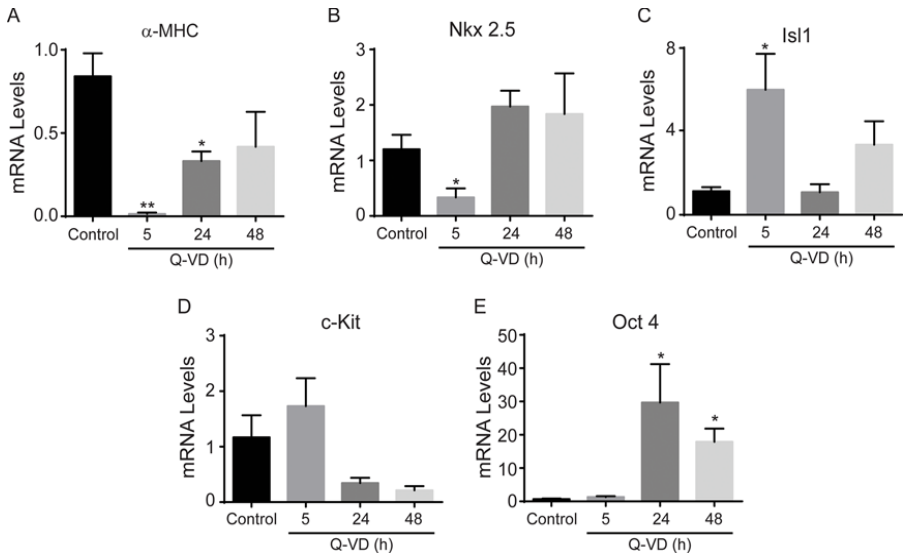

Figure 8. Caspase inhibition suppresses cardiac differentiation

When a broad- spectrum caspase inhibitor Q-VD-OPH was added to the differentiating EB cultures we found that the levels of α-MHC (A) and Nkx2.5 (B) significantly decreased by acute Q-VD-OPH treatment (5h), while chronic treatment (24 h and 48 h) only affected α-MHC expression. In contrast, Isl1 expression (C) was strongly up-regulated by acute Q-VD treatment.

A mechanism of action by which active caspase 3 induces cardiac differentiation might be through cleavage and activation of mitogen-activated protein kinase (MAPK) signaling cascades (MST1/MKK6/p38) (Fernando *et al.* 2002). Downstream components of these cascades up-regulate Mef2a and c factors known to be important for cardiomyocyte differentiation (McKinsey *et al.* 2000). In the work by Abdul-Ghani et al. (Abdul-Ghani *et al.* 2011), the authors demonstrated that caspases have a role in cardiac differentiation mediated through their proteolytic cleavage of β-catenin, leading to a loss of canonical Wnt signaling activity (Steinhusen *et al.* 2000). Whereas the response to injury is intensively studied in the adult heart, much less is known about response to apoptotic stimuli in embryonic cardiomyocytes. Recently, a systematic and comprehensive characterization of stress response, using a model of tissue mosaicism for mitochondrial dysfunction in prenatal mouse hearts, showed an impressive ability of embryonic cardiomyocytes to adapt to stress (Magarin *et al.* 2016), which is in line with our observations. However, additional studies are necessary to determinate the exact cell populations responsive to caspase activation as well as to further dissect the mechanism of action of caspases in the embryonic heart.

Taken together, our findings provide insights into the fate decisions that regulate cell death or differentiation, by which stem cells with cardiac differentiation potential may become activated following injury.

4.2 **STUDY II**

The optimal cells for heart regeneration should be able to proliferate in damaged myocardium while differentiating into endothelial cells, smooth muscle, and cardiomyocytes. In Study II we describe that by culturing fetal cardiac MSCs on biologically relevant laminins (LN) in a medium containing molecules stimulating the Wnt/β-catenin pathway we can generate cells with cardiac progenitor characteristics. Furthermore, this culture system promotes large-scale production of cardiac MSCs, while retaining their cardiovascular progenitor phenotype, with low gene-expression variability during the course of culture. The gene-expression profile of the human fetal cardiac MSCs showed core group of transcription factors known to be important in the regulation of cardiogenesis, including the expression of mesodermal markers: Tbx6, Tbx1/Brachyury, Tbx18, and Mesp1; key transcription factors involved in the SHF development in mice (Black 2007): Isl1, Gata4, Nkx2.5, Foxh1, Fgf8; the FHF (Tbx5, Nkx2.5), as well as other markers related to cardiovascular progenitors like Pdgfrα and the stemness markers Kdr, Ssea-1, and c-Kit. However, the cultured cardiac MSCs also expressed mature cardiomyocyte markers such as myosin heavy chain (Myh) 6 and 7, the endothelial marker Vcam1, as well as the smooth muscle markers Myh11 and myocardin (Myocd). These cells can be directed towards cardiomyocytes using LN-211 and can also be differentiated into endothelial cells and smooth muscle cells (Figure 9).

Interestingly, the use of specific LN molecules supported lineage conversion of the cultured cardiac MSCs into cardiomyocytes and endothelial cells. Using LN-211 alone, the main component of the basement membrane surrounding adult cardiac and skeletal muscle fibers in situ (Domogatskaya *et al.* 2012), stimulated cardiac commitment of fetal cardiac MSCs at the gene-expression level, with a 150 times increase of TnT expression and a concomitant up-regulation of Nkx2.5. In addition, culture on LN-521/411, which is present in the basal membranes of vessels (Hallmann *et al.* 2005, Simon-Assmann *et al.* 2011, Domogatskaya *et al.* 2012), supported differentiation toward endothelial cells expressing CD31.

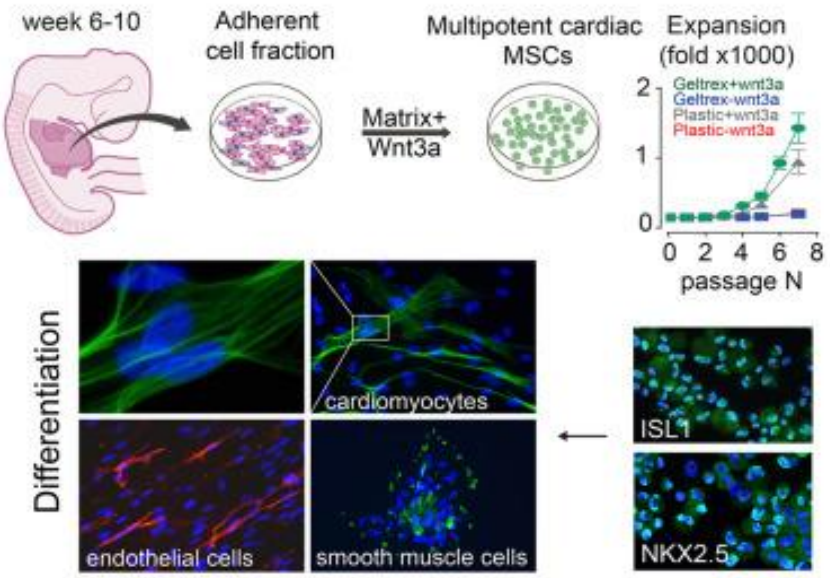

Figure 9. Derivation of human fetal cardiac MSCs with cardiac progenitor characteristics

In order to develop a culture protocol for successful propagation of cardiac MSCs, we started from the adherent cell fraction from whole human fetal hearts (gestational weeks 6–10). At 2 weeks, the adherent cells had expanded more than 1,000-fold and demonstrated activation of transcription factors involved in defining cardiac progenitors and multi-potent stem cells. After 3 weeks in culture the majority of cardiac MSCs stained positive for Isl1 (>90%) and Nkx2.5 (>80%). A portion of the cultured cardiac MSCs derived from the 9-week heart differentiated within three weeks into spontaneously beating, TnT+, striated cardiomyocytes. Endothelial cell differentiation was induced in cardiac MSCs by using a combination of medium containing vascular endothelial growth factor (VEGF) and LNs present in the basal membranes of blood vessels (LN- 521 and LN-411) as substrata. After 3 weeks in culture, a small proportion of the cardiac MSCs expressed the endothelial marker CD31. The fetal cardiac MSCs expressed α-SMA prior to differentiation. Interestingly, after smooth muscle cell differentiation, the α -SMA polarized into podosome-like structures, which can be found in vascular smooth muscle cells upon stimulation with exogenous PDGF-BB and TGF-ß.

These characteristics of fetal cardiac MSCs indicate their plasticity as well as the potential use of combinations of these cells, specific laminins and signaling molecules for generation of cardiac progenitor cells for cardiac repair.

The origin of the different subpopulations of cardiac MSCs has not been examined in this study. In the present study, the majority of the cultured cardiac MSCs stained positive for Pdgfrα. Whether these cells possibly originate from interstitial cells of the atria and ventricles remains to be elucidated, but they displayed a similar capacity to

differentiate into endothelial cells and also cells with polarized α -SMA expression akin to that of mature smooth muscle cells. In contrast to the Pdgfrα+ cells derived from second trimester hearts (Chong *et al.* 2013), the fetal cardiac MSCs derived from a 9-week human heart could be differentiated into spontaneously beating, striated TnT+ cardiomyocytes. However, the cells derived from 6- to 8-week human fetal hearts did not differentiate into TnT+ cardiomyocytes, despite similar expression of Isl1, Nkx2-5, Pdgfrα, Ssea-1, Kdr, and c-Kit before initiation of differentiation. This restricted differentiation capacity might be related to the age of the fetal heart from which the cardiac MSCs were derived. However, age-related differences in expression of markers known to characterize cardiovascular progenitors were not observed in this study. Some of the cultured cardiac MSCs also stained positive for the mesodermal progenitor marker Tbx18 (Christoffels *et al.* 2006, Mommersteeg *et al.* 2010) and future studies need to be performed in order to address whether some of the Pdgfrα + and Tbx18+ cells potentially share a developmental origin.

4.3 **STUDY III**

In Study III, we performed bioenergetic profiling of cardiac MSCs derived from human fetal hearts from gestational weeks 4.5 to 10.5 and cultured under standard conditions in normoxia. The basal and ATP-linked (mitochondrial) respiration, as well as the maximal respiration levels increased significantly over time during the first trimester indicating that active OxPhos is likely required to meet high ATP demands needed for extensive biosynthesis during fetal heart development.

Mitochondria are known to change morphology in accordance with the metabolic needs of the cell. We observed predominantly perinuclear accumulation of mitochondria in human fetal cardiac MSCs with a distinctive crypta formation and elongated morphology. Mitochondria in the cardiac MSCs derived from the 5th gestational week are presented as separate organelles, while instead, mitochondria of MSCs derived from hearts from the end of the first trimester formed a network. Mitochondrial network formation indicates mitochondrial activation, which is in accordance with our bioenergetic profiling data that shows increased OxPhos in later gestational weeks.

In Study II, the cells derived from a 9-week heart showed most pronounced cardiogenic capacity and were able to give rise to spontaneously beating cardiomyocytes. Accordingly, in Study III, human fetal cardiac MSCs derived from the same 9-week

heart that previously demonstrated cardiogenic capacity, showed an increase in mitochondrial OxPhos after three weeks of stimulation with Wnt3a. This change might support the propagation of cells with cardiac progenitor characteristics from the MSCs of the 9-week heart, since more effective ATP production is crucial for contractile cells. The developmental dynamics of the glycolytic to OxPhos transfer network is distinctive for the remodeling of cellular energetic infrastructure underlying stem cell cardiogenesis (Chung *et al.* 2007, Ellen Kreipke *et al.* 2016). Thus, the human fetal cardiac MSCs´ ability to respond to prolong Wnt3a stimulation with increased OxPhos activation seems to be predictive for their cardiac lineage commitment.

This observation warrants further investigation as it may provide clues for developing new approaches that will allow manipulation of the fate and lineage specification of the MSCs, in a similar way to the recent mass production of functional cardiomyocytes from human ESCs achieved by changing the primary carbon source in growth media from glucose to lactate (Tohyama *et al.* 2013).

In conclusion, our findings provide important insights on the distinct bioenergetics of fetal cardiac MSCs during heart development. Optimal metabolic conditions do not only need to be permissive for the expansion and directed differentiation of cells, but could also serve as instructive regulatory signals leading to development of new drugs.

5 CONCLUSIONS

I. Sublethal caspase activation increases the yield of early cardiomyocytes derived from mouse ESCs, whereas caspase inhibition decreases cardiomyogenesis

II. Multi-potent human cells with cardiac progenitor characteristics can be propagated from MSCs of fetal hearts by using a combination of canonical Wnt/β-catenin stimulation and specific laminin isoforms as cell culture substrata

III. Metabolomic profiling of human fetal cardiac MSCs shows increase in oxidative phosphorylation during first trimester development as well as during their expansion towards multi-potent cells with cardiac progenitor characteristics.

6 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Regenerative cardiology that initially aimed to directly replace lost cardiomyocytes with exogenous stem cells has undergone a profound paradigm shift, leading us to consider that therapeutic activity reflects primarily an indirect, paracrine effect of delivered cells to trigger an endogenous regenerative cascade. However, the incidence of reparative stem cells with a clinically measurable cardio-regenerative capacity is rather rare in patient cohorts (Cogle *et al.* 2014). Patient's age, sex, morbidities, and concomitant therapies may impact regenerative potential. It has been suggested that non-regenerative cells remain confined to a state of perpetual stemness (Marban and Malliaras 2012), whereas regenerative cells are environment responsive, plastic, with an inclination towards cardiovascular lineage specification (Behfar *et al.* 2010, Terzic and Behfar 2016).

Bone marrow-derived MSCs represent a rare population of multi-potent stem cells, capable to differentiate into osteoblasts, adipocytes, chondrocytes, muscle cells and stromal fibroblasts (Friedenstein *et al.* 1968, Caplan 1991, Prockop 1997, Pittenger *et al.* 1999). MSCs feature several clinically relevant advantages such as ease of isolation, high expansion potential with stable gene expression as well as immunomodulatory capacities (Le Blanc *et al.* 2008).

However, there are also major limitations related to BM-MSCs, for instance their tendency to lose clonogenicity and differentiation capacity when expanded *in vitro*, which might affect the outcome upon use in clinical trials. Age of the donor, plating density, serum composition and passage time have all been implicated in MSCs senescence. In order to by-pass these issues, it is important to continue developing expansion protocols of MSCs to preserve their multi-potency and immunomodulatory ability.

In the POSEIDON randomized trial where autologous and allogeneic MSCs were compared in patients with ischemic cardiomyopathy, these cells demonstrated limited effect on the functional capacity as well as the remodeling of the left ventricle (Hare *et al.* 2012). This trial proved the safety of allogeneic MSCs for clinical use, as previously demonstrated in studies in graft versus host disease (Le Blanc *et al.* 2008).

One strategy to make implanted MSCs more homogenous is to prime them into cardiopoietic MSCs. Using the same embryonic signals that instruct precardiac

mesoderm into the cardiomyogenic fate (Behfar *et al.* 2008), it is possible to impose a lineage-specifying program on stem cells (Behfar *et al.* 2010). In a clinical phase I/II trial (C-Cure trial) in patients with ischemic cardiomyopathy, the cell-treated group demonstrated improved EF as well as functional capacity in comparison to the control group (Bartunek *et al.* 2013). However, these effects were not reproduced in the European phase III multi-center trial CHART-1. (http://www.celyad.com/news/celyad-announces-results-for-the-chart-1-phase-iii-clinical-trial-evaluating-c-cure-cell-therapy).

Based on previous studies, the optimal cells for heart regeneration should be able to survive in the injured myocardium while exerting beneficial effects on the surrounding cells by stimulating angiogenesis and inhibiting apoptosis of cardiomyocytes. In paper II, we have developed a protocol to derive and expand human fetal cardiac MSCs with characteristics of multi-potent cardiac progenitor cells. We used biologically defined culture conditions by employing specific laminins in combination with canonical Wnt/ β-catenin stimulation.

Based on our preliminary results, when using the same protocol on the adult BM-MSCs, these culture conditions enhanced proliferation, clonogenicity, multi-potency, as well as immunomodulatory and cardiogenic lineage specification, all of which may significantly improve the therapeutic potential. If our concept is reproducible in mature BM-MSCs, it would enable us to expand allogeneic BM-MSCs into large number of cells with cardiac progenitor characteristics to be used at the time of myocardial infarction, preventing the remodeling process and the development of heart failure. Beyond traditional focus on induced expression of cardiac specific transcription factors, to direct the stem and progenitor cells towards cardiovascular fate, the recent studies of stem cells metabolism are revealing clues about potential metabolic signatures distinctive for cells with higher regenerative capacity and cardiogenic potential.

In study III we suggest that a deeper understanding of metabolism in quiescent stem cells is necessary to define optimal *in vitro* conditions for expansion and differentiation of MSCs, improving survival and engraftment *in vivo*. These strategies might be important to define the criteria to choose the MSCs with the best regenerative potential to be used in clinical trials. In a clinical setting, the optimal metabolic state of the implanted cells might differ if these cells are used for treatment in the acute or chronic phase of heart failure.

In the MSCs derived from fetal hearts during the first trimester of gestation, we observed increase in aerobic mitochondrial metabolism. Furthermore, stimulation of the

canonical Wnt/ β-catenin signaling pathway in hypoxia appeared to enhance long-term MSC expansion and resulted in a cell population with higher cardiogenic differentiation potential.

The embryonic heart has an impressive regenerative capacity (Sturzu *et al.* 2015, Magarin *et al.* 2016) and Porrello and colleagues have demonstrated that the mouse heart can still fully regenerate if amputation of the ventricular apex occurs on the first neonatal day (Porrello *et al.* 2011). However, this initial robust regenerative potential is rapidly lost already within the first postnatal week (Porrello *et al.* 2011). Interestingly, immediately after birth the cardiomyocytes produce more than half of their ATP through glycolysis, while at seven days after birth, glycolysis decreases accounting for less than 10% of the ATP production (Lopaschuk and Jaswal 2010). Recent report from Puente et al. indicated that the increase in environmental oxygen, and the subsequent upregulation of oxidative metabolism is the upstream signal that triggers cell cycle exit of cardiomyocytes shortly after birth (Puente *et al.* 2014). Additionally, by fate mapping of hypoxic cells, Kimura et al. identified a rare population of hypoxic cardiomyocytes in the adult mice that display characteristics of proliferative neonatal cardiomyocytes (Kimura *et al.* 2015). These results suggest that hypoxia signaling is an important hallmark of cycling cardiomyocytes. Indeed, distinct metabolic signature of fetal cardiac cells may contribute to remarkable regenerative ability of the fetal and neonatal heart.

Based on these findings it is tempting to speculate that, the reactivation of fetal survival mechanisms in the adult heart represents an attractive therapeutic goal.

Mature cardiomyocytes were shown to have a capacity to dedifferentiate in response to specific stimuli (Kubin *et al.* 2011) and differentiated, multinucleated skeletal muscle cells can be dedifferentiated into proliferating progeny by first inducing and subsequently intercepting a programmed cell death response (Wang *et al.* 2015). Recently, the role of caspases in promoting differentiation of embryonic stem cells (Fujita *et al.* 2008) made these proteases attractive candidates in regulating differentiation rather than just executers of programmed cell death. In paper I, we described that a sublethal apoptotic stimulus triggers a caspase-dependent signaling mechanism and increases the yield of early cardiomyocytes derived from mouse ESCs (Bulatovic *et al.* 2015). In a clinically relevant setting, our results suggest that caspase-controlled pathways may have a role in bridging an acute cell injury to the fate decision of exogenous and endogenous cell sources. It was previously reported that the exposure of cells to sublethal hypoxia increased the their tolerance to the harsh environment after

transplantation (Yu *et al.* 2013). These preconditioned cells also showed increased differentiation, enhanced paracrine effects leading to increased trophic support, and improved homing to the site of the lesion (Yu *et al.* 2013). If the induction of sublethal apoptotic stimuli can stimulate generation and proliferation of new cardiomyocytes in human embryonic stem cells and *in vivo* after a myocardial infarction and if this approach can be combined with cellular therapy or delivery of drug-loaded ECVs needs to be further explored.

In conclusion, by creating reliable maps of cardiovascular progenitors and their metabolic state, it may become possible to generate highly expandable cells with cardiac progenitor properties from adult MSCs, while preserving their immunomodulatory characteristics and create an off-the-shelf system for use in an acute and chronic setting.

Finally, this thesis explores a role of apoptotic stimuli and metabolic flexibility in cardiac progenitor fate determination. The future approach based on deeper understanding and targeted manipulation of these signals may enhance the heart's intrinsic regenerative capacity through activation of endogenous progenitor cells or stimulation of cell cycle reentry in adult cardiomyocytes.

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